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EFFECTS OF PHARMACOLOGIC INTERVENTION ON OXYGENATION, LUNG WATER AND PROTEIN LEAK IN THE PSEUDOMONAS ARDS PROCINE MODEL

SUBTITLE: EFFECTS OF PHARMACOLOGICAL AND IMMUNOLOGICAL INTERVENTION ON A PORCINE PSEUDOMONAS MODEL OF SEPSIS INDUCED ADULT RESPIRATORY DISTRESS SYNDROME (AIDS)

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Adult respiratory distress syndrome (ARDS) is an explosive form of acute respiratory failure, most commonly seen in critically-ill patients (Byrne et al. 1987). It is characterized by clinical respiratory distress, marked hypoxemia refractory to increased F_1O_2 and, bilateral infiltrates on chest x-ray in the absence of increased left atrial filling pressures. Histologically, ARDS is characterized by engorgement of pulmonary capillaries with inflammatory cells and the presence of large amounts of hyaline material in the alveoli with evidence of widespread injury to the alveolar-capillary membrance. It is this latter feature of diffuse alveolar-capillary membrane injury with consequent increased permeability pulmonary edema which distinguishes ARDS from other causes of acute respiratory failure, such as acute alveolar hypoventilation due to respiratory center depression or neuromuscular disease, acute hypoxemia associated with congestive cardiac failure, reduced functional residual capacity, commonly seen in the post-operative surgical patient, severe asthma, pulmonary embolism and bacterial pneumonia.

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Introduction

Adult respiratory distress syndrome (ARDS) is an explosive form of acute respiratory failure, most commonly seen in critically-ill patients (Byrne et al.1987). It is characterized by clinical respiratory distress, marked hypoxemia refractory to increased F_IO_2 and, bilateral infiltrates on chest x-ray in the absence of increased left atrial filling pressures. Histologically, ARDS is characterized by engorgement of pulmonary capillaries with inflammatory cells and the presence of large amounts of hyaline material in the alveoli with evidence of widespread injury to the alveolar-capillary membrane. It is this latter feature of diffuse alveolar-capillary membrane injury with consequent increased permeability pulmonary edema which distinguishes ARDS from other causes of acute respiratory failure, such as acute alveolar hypoventilation due to respiratory center depression or neuromuscular disease, acute hypoxemia associated with congestive cardiac failure, reduced functional residual capacity, commonly seen in the post-operative surgical patient, severe asthma, pulmonary embolism and bacterial pneumonia.

Risk Factors For ARDS (Table 1)

Bacteremia/Endotoxemia
Aspiration
Pancreatitis
Inhalation of Smoke or Toxic Gas
Burns
Severe Trauma
Anaphylaxis
Embolism

The definition of ARDS has posed a problem since it was described by Ashbaugh and Petty in 1967 (Ashbaugh et al.1967). The approach employed by this group - description of the clinical syndrome with exclusion of other possible causal factors - made ARDS a diagnosis of exclusion. In more recent times, ARDS has been viewed as representing a spectrum of lung injury. Therefore any definition that employs specific blood gas or pulmonary artery occlusion pressure values may not

include all patients having some degree of lung injury. The task force on respiratory disease defined ARDS as *"acute respiratory failure and distress associated with a specific incident or illness"*. This leads to further difficulty in the concise definition of ARDS as many conditions have been shown to predispose to or complicate ARDS. ARDS may thus be said to represent the response of the lung to a specific insult, though this insult is often difficult to identify and a cause and effect relationship even more difficult to demonstrate. Nevertheless, the term ARDS is usually reserved for patients who develop a threshold level of lung injury, so that comparisons between different groups of patients and between patients in different institutions may be made.

Criteria for diagnosis of ARDS (Table 2)

Clinical Respiratory Distress
Respiratory Rate > 20
Severe Hypoxemia
PaO2 / FiO2 < 150 Without PEEP
PaO2 / FiO2 < 200 With PEEP
Diffuse Bilateral Infiltrates on Chest X-Ray
Presence of Known Risk Factor For ARDS
Optional
Decreased Respiratory Compliance
Pulmonary Wedge Pressure < 18 mmHg

Currently, definitions of ARDS include a degree of hypoxemia, chest radiograph criteria, clinical respiratory symptoms or signs in the presence of a known predisposing risk factor. Generally accepted criteria for the diagnosis of ARDS are summarized in table 2. The incidence of ARDS has been estimated to be of the order of 150,000 cases per year in the USA and it carries an overall mortality of 60-70%. In a study from the National Lung, Heart and Blood Institute, the only two factors that correlated with survival were age and the presence of multisystem organ failure. In patients with acute respiratory failure only, mortality was 41%. Mortality progressively increased with increasing number organ sytems failing, such that mortality was 100% in patients with failure of 4 or more organ systems. The combination of respiratory and renal failure,

regardless of other organ involvement was associated with a mortality rate of 85%. Early research efforts focused on the disruption of alveolar function seeking to re-establish alveolar expansion, reduce accumulation of fluid in the alveoli and improve gas exchange. Despite advances in anesthetic technique, improvements in pharmacologic agents and intensive care, the mortality associated with ARDS has hardly changed in the past 25 years. ARDS thus remains a formidable clinical problem in both military and civilian medical practice.

Though Ashbaugh and Petty were the first to coin the term "ARDS", to ensure increased awareness of ARDS in civilian medical practice, the concept of diffuse lung injury subsequent to sepsis or injury elsewhere had long been noted in the military surgical community. A condition known as the traumatic wet lung syndrome was described in combat soldiers during World War II. The problem of "wet lung" was also noted to be present in soldiers who had sustained non-thoracic trauma such as abdominal wounds, fractures of long bones and head injuries as well those with thoracic injuries. In a report (Grant and Reeves, 1951) on all injuries treated during World War II, Grant and Reeves noted "*Of all the organs, the lungs were the most frequently abnormal (at autopsy). ....they displayed varying degrees of congestion and edema, often with petechial or larger hemorrhages and more or less widespread bronchopneumonia in addition.*" By the time the Vietnam war came to be fought, the concept of rapid evacuation of wounded soldiers from the field and early definitive treatment of their injuries had been established. This saved many lives but, as a contemporary observer remarked "*A new type of surgical patient has appeared in Vietnam which is of great importance to surgical research. This is the critically wounded patient who is suffering rapid blood loss from vascular or organ injury, who under any other circumstances would have died shortly after injury. However he is now delivered to a hospital frequently within 15 minutes of injury. ...he has a pO_2 of 40 but there may be no clinical evidence for this and no respiratory injury.*"

At the time of writing of this report, it is now known that the association of sepsis and ARDS is of particular importance as together with trauma and aspiration these causes account for almost 75% of all cases of ARDS (Fowler et al.1983). Further, sepsis in the setting of ARDS either as a cause or complication carries a particularly high mortality. In recent years, with increased knowledge of the pathophysiology of acute lung injury and derangements in microvascular function and the role of complement, eicosanoids and cytokines such as tumor necrosis factor-α (TNFα) and interleukin-1 (IL-1), therapeutic intervention has begun to focus on inhibition or blockade at pivotal sites in the complement, eicosanoid and cytokine cascades, in an attempt to modify the host response to injury and attenuate or prevent end-organ damage such as that seen with ARDS.

In the fully developed state, ARDS is a protein rich inflammatory pulmonary edema with sequestration of neutrophils, initially in the pulmonary vasculature but later in the interstitium and the alveolar space. The mechanisms that lead to the development of this protein rich inflammatory edema are complex. Many substances have been implicated as either triggers or mediators of a cascade of events which, once started, spins rapidly out of control. Those substances implicated include endotoxin (Bloom et al.1988), complement (Brigham et al.1974), eicosanoids (Hales et al.1981) and cytokines such as TNFα, IL-1, and IL-6 (Beutler and Cerami, 1987; Tracey et al.1986; Cannon et al.1990; Kelley, 1990). It has long been speculated that, of the cells mediating acute lung injury, the neutrophil (PMN) is the one most central to the disease process (Windsor et al.1993; Tate and Repine, 1983; Staub et al.1982). This was first postulated by Metchinkoff in 1887. Since then the role of the PMN in inflammatory mediated host injury has become increasingly certain. Despite isolated reports that neutropenic septic patients are still capable of developing an acute lung injury in response to sepsis (Ognibene et al.1986; Maunder et al.1986) reperfusion, aspiration etc, the weight of evidence implicates the PMN as critical to producing

ARDS. A number of observations support this theory: post-mortem histological evidence of PMN sequestration in the lungs of patients who have died from ARDS (Orell, 1971) as well as recovery of large numbers of PMNs from bronchoalveolar lavage (BAL) of patients early in the course of ARDS (Fowler et al.1987) in addition to PMN secretory products such as elastase in the same lavage fluid (Lee et al.1981). Correlations exist between the appearance of the PMNs in the BAL and the acute neutropenia reported in numerous animal models of sepsis induced acute lung injury (Meyrick and Brigham, 1983; Walsh et al.1991).

The presence of PMNs in the lung, however, does not necessarily incriminate them as the primary effector cells. Fowler et al noted that patients (Fowler et al.1987) at high risk for developing ARDS, but in whom the fully established syndrome did not materialize, still had large numbers of PMNs in the lavage fluid. This may suggest that PMNs are merely markers of lung injury and not the primary effector. To refute this notion, however, many reports show that pre-insult PMN depletion prevented the evolution of an acute lung injury (Stephens et al.1988; Helfin and Brigham, 1981).

In 1910, Andrews speculated that ***"if the bone marrow is the birthplace of the PMN and the spleen is their ultimate tomb, while the blood is their means of transit, the lungs may serve as a weekend retreat where they may recuperate their energies"***. This quote defines the equilibrium that exists between the circulating PMNs and those PMNs that are in the so called 'marginating pool' present, in loose association with the pulmonary endothelium by the action of a divalent cationic-dependent process. In order to respond to the invasion of pathogens, a change in the loose association of the marginating pool must take place to allow the PMNs to be sequestered and then kill. This change is manifest by neutrophil chemotaxis, adherence and finally migration across endothelial layers. Local and systemic release of chemical mediators, including cytokines, complement protein-degradation products, eicosanoids and platelet activating factor initiate this sequence. Mediators act first as

chemoattractants recruiting PMNs from the circulating pool and second to activate both the PMNs and the endothelial cells to express a complex sequence of surface protein receptors whose action is to promote adherence and migration.

The transformation from a marginating to an adherent PMN is based on a two step theory (Kishimoto, 1991). Inflamed endothelium expresses the endothelial-leukocyte adhesion molecule (E-Selectin), the ligand for which is a member of the selectin family of adhesion receptors on unstimulated PMNs, designated leukocyte endothelial cell adhesion molecule (L-Selectin). This receptor/ligand binding initiates PMN rolling along endothelial surfaces. Now, bathed in a complex milieu of chemical mediators of inflammation, the PMN is activated and upregulates the Mac-1 (CD11b/18) member of the CD11/18 family of integrins, increasing the number and configuration of this surface protein receptor. Upregulated CD11b and constitutively expressed CD11a receptors bind to increasingly upregulated intercellular adhesion molecule (ICAM-1) expressed on the inflamed endothelium. This ligand/receptor complex provides strong adhesive forces and is necessary for transmigration of the PMN across the endothelium. Coincident with rapid upregulation of CD11b receptors there is a rapid shedding of the L-selectin receptor allowing, unopposed PMN migration.

Neutrophil activation and receptor expression is accompanied by an outpouring of PMN proteases and generation of reactive oxygen metabolites (ROM)(Solomkin et al.1985; Weiss, 1989; Bretz and Baggiolini, 1974). With activated PMNs tightly attached to the endothelium, they are thus in a position to produce the alveolar-capillary membrane injury typical of ARDS. This hypothesis of acute lung injury holds that neutrophil tissue injury is preceded by neutrophil adherence to endothelium. ROM and proteases are released into a protected microenvironment generated by the adhesion of the neutrophil to the endothelium, isolated from the effects of free oxidant scavengers and protease inhibitors. This results in localized high concentrations of toxic metabolites and thus, localized endothelial damage.

The neutrophil does not act in isolation during the initiation and propagation of this acute lung injury (Rinaldo and Christman, 1990). Monocytes and alveolar macrophages also release ROM and vasoactive substances such as arachidonic acid metabolites which directly affect pulmonary and systemic vascular tone. In addition, these cells generate large quantities of cytokines such as TNFα, IL-1 and IL-6. In recent years, studies from our laboratory and others have shown that cytokine networks play a central role in the initiation and propagation of the physiologic and pathologic events seen in acute lung injury (Molloy et al.1993), acting as intercellular signalling systems, recruiting inflammatory cells to an injured area and modulating the function of cells, so recruited (Mantovani and Dejana, 1989).

The association of neutrophil activation and microvascular endothelial injury has become a major focus of research in this laboratory in recent years as we have sought to attenuate acute lung injury either by altering neutrophil function or by altering PMN adhesion to vascular endothelium.

Material And Methods

The Porcine Model

The porcine model was used in all experiments. Swine weighing between 15-25 kgs were anesthetized with intramuscular ketamine hydrochloride 25 mg/kg and placed supine. Anesthesia was induced with sodium pentobarbital (10 mg/kg) and maintained with an infusion of pentobarbital and fentanyl at a rate of 5-10 mg/kg/min and 2.5-7.5µg/kg/min respectively Earlier studies were performed with paralyzed animals, since alveolar-capillary protein leak was measured with a computerized gamma camera in which the animals could not be allowed to move. Paralysis made evaluation of adequate anesthesia difficult and was therefore discontinued in later studies. Following intubation with a cuffed endotracheal tube, the animals were ventilated with an F_iO_2 of 0.5 at a positive end expiratory pressure (PEEP) of 5 cm H_2O and a tidal volume of 12-15cc/kg at a rate to produce a $PaCO_2$ of approximately 40 torr at the beginning of the experiment.

Live *Pseudomonas aeruginosa* (PAO strain, 5×10^8 CFU/ml at 0.3 ml/20kg/min) was then administered for 1 hour. In pseudomonas (Ps) infused animals this produces a marked physiological deterioration, representative of acute ARDS, resulting in immediate, significant increase in pulmonary artery pressure (PAP) which persists throughout the entire duration of the experiment. Systemic arterial pressure (SAP) shows a progressive decline as does cardiac index and PaO_2. Extra-vascular lung water (EVLW) becomes significantly elevated when compared to saline infused controls.

Catheters were inserted into the left common carotid artery for monitoring SAP and arterial blood gas determinations, and into the right and left external jugular veins for infusion of *Pseudomonas* and the therapeutic agents to be studied. A Swan-Ganz catheter was passed from the right jugular vein into the pulmonary artery and wedged into a small branch with the balloon inflated, using pressure monitoring. It was thus

possible to record pulmonary artery pressure (PAP), pulmonary capillary occlusion pressure (PCOP) and cardiac output, using a thermodilution technique. Cardiac output is converted to cardiac index (CI) by the formula:

$$\frac{CO}{0.112\ BW^{2/3}}$$

where BW is the body weight in kg.

Arterial and mixed venous blood gases were analyzed using a blood gas analyzer (Instrumentation Laboratories, Model 113).

A 5 French femoral artery lung water catheter (American Edwards Laboratories, Model 96-020-5F) was passed into the lower abdominal aorta for measurement of extravascular lung water (EVLW) using a thermal dye dilution technique. In this technique, 10 ml of iced, green dye solution (2 mg indocyanine green dye in 10 ml 5% dextrose) are injected as a bolus through the proximal port of the Swan-Ganz catheter as blood is simultaneously withdrawn through the thermistor-tipped femoral artery catheter connected to a densitometer cuvette (Waters Instruments In., Model 402A) which is linked to a lung water computer (American Edwards Laboratories, Model 9310). The computer measures the mean transit times of the intravascular dye (MTD) and freely diffusible thermal component (MIT) as well as the cardiac output (CO). EVLW is calculated by the formula:

$$\frac{CO\ (MTD-MIT)}{BW\ (kg)}$$

Bronchoalveolar Lavage and Protein Assay

Using a fiberoptic bronchoscope (Machita VT-5100C, 4mm) bronchoalveolar lavage (BAL) was performed at 0 and 300 min. The middle and lower lobes of the right lung were lavaged (4 x 25 ml aliquots of sterile 0.9% NaCl in each lobe) at 0 min. This was repeated in the corresponding lobes of the left lung at 300 min. Lavage returns

were consistently high (>75%) in all animals. BAL protein content, expressed as micrograms protein per milliliter of recovered lavage fluid, was performed on non-cellular fractions of the BAL by a modification of the Lowry technique.

Peripheral Blood Samples

Peripheral blood samples for isolation of neutrophils and subsequent assay of short and long-lived toxic oxygen metabolites were withdrawn at baseline (zero timepoint), and at 5 hours (end-stage sepsis). The neutrophils were isolated using dextran sedimentation and Ficoll-hypaque density gradient centrifugation. Assays of phorbol ester stimulated production of oxygen-dependent neutrophil products were performed as described later in this report. Similarly, arterial blood samples were drawn at hourly intervals for estimation of neutrophil counts, thromboxane B_2 levels, TNFα and IL-6 and also for measurement of CD18 receptor expression on peripheral blood neutrophils.

Measurement of CD11/CD18 Receptor Expression

Saturating concentrations of fluorescein-conjugated monoclonal antibodies specific for the ß-subunit of the CD18 glycoprotein were incubated with neutrophils for 20 min at 4°C. To control for non-specific binding, the same concentration of fluorescein-conjugated murine IgG_{2a} was incubated with identically treated neutrophils. Cells were washed thoroughly and fixed in 1ml paraformaldehyde. Immunofluorescence intensity was analyzed with a flow cytometer equipped with a logarithmic amplifier. The channel number (log scale) representing the peak fluorescence intensity of 5,000 cells was determined. Linear fluorescence-intensity was calculated from a logarithmic-linear calibration curve. Final expression of CD18 was calculated by subtracting the linear fluorescence intensity of the bound non-specific IgG_{2a} from the linear fluorescence intensity of the bound MoAb 60.3.

Tumor Necrosis Factor Activity

Arterial blood samples were collected at baseline, 30 min and then at 60 min

intervals for measurement of plasma TNFα activity. The mouse L929 fibroblast bioassay was used to quantify TNFα activity (Flick and Gifford, 1984). Arterial blood samples were drawn into sterile glass tubes containing 0.15% EDTA and kept at 4°C. Specimens were centrifuged at 500 g for 20 min at 4°C and the resulting plasma frozen at -20°C until time of assay. L929 cells were seeded into flat-bottomed 96 well microtiter plates (Corning, NY) at a density of 4×10^4 cells/well and grown to confluence overnight in Dulbecco's minimal essential medium (GIBCO) containing 1% penicillin-streptomycin and 5% fetal calf serum (DMEM). Medium was then removed from confluent monolayers and 100 µl of DMEM containing Actinomycin-D (Merck, Sharp and Dohme, Westpoint, PA, final concentration = 5ug/ml) were added to each well. One hundred µl of each of the following were then added to selected duplicate wells containing L929 cells: 1) DMEM (0% cytotoxicity); 2) serial dilutions of recombinant TNFα (5×10^{-3} to 6×10^{-4} U/ml) (Cetus Corp., Emeryville, CA); 3) plasma samples from different groups; 4) DMEM in blank wells without cells (100% cytotoxicity). Plates were then incubated for 20 hr at 37°C in 5% CO_2. Following incubation, the medium was removed and the L929 cells were stained for 10 min with 0.5% crystal violet in 20% methanol, rinsed in water and air dried. Optical density (OD) of each well was determined by a microplate reader (Bio-Tek EL 309) and calibrated to non-cellular reagent blanks at a wavelength of 550 nm. The percent cytotoxicity of L929 cells was calculated by :

$$\% \text{ Cytotoxicity} = \frac{\text{OD wells with 0\% cytotoxicity - OD experimental sample well}}{\text{OD wells with 0\% cytotoxicity}}$$

TNFα activity was expressed in units per milliliter (U/ml), where one unit of TNFα activity is defined as 50% L929 cytotoxicity.

Superoxide anion kinetics assay

Spontaneous and phorbol myristate acetate (PMA) stimulated generation of superoxide anion was measured in freshly harvested neutrophils, by continuously monitoring superoxide dismutase (SOD) inhibitable reduction of ferricytochrome C using a dual beam spectrophotometer (Shimadzu UV-160). Briefly, 650 µl of neutrophil suspension (4.0 x 10^6 cells/ml, in PBS) was added to a reaction mixture in a flat bottomed cuvette (Fisher Scientific). The reaction mixture contains 200µl of stock ferricytochrome C solution (16 mg/ml, Sigma chemical company) in a volume brought to 2.6 ml with PBS. Reference cuvettes contained cells plus cytochrome C plus superoxide dismutase (100µg/ml). Cuvettes were permitted to equilibrate in the spectrophotometer at 37°C under continuous stirring for ten minutes. PMA (2.6 µl of 100 ng/ml stock) was then added to give a final cuvette concentration of 200 nmol/ml. The change in optical density was continuously recorded at 550 nm for a 10 minute period. Assays were performed in triplicate. The nanomolar extinction coefficient of 0.0211 for the reduction of ferricytochrome C was used to quantify superoxide anion production. Superoxide anion production, expressed as nmols of O_2^-/min/10^6 PMN, was calculated for each minute and plotted against time .

Measurement of Hypochlorous acid production.

Neutrophil HOCl production was determined by a modification of the chlorination of taurine method where the ability of the amino acid taurine to act as a scavenger of HOCl is utilized. The resulting chlorination complex (taurine chloramine), oxidizes 5-thio-2-nitrobenzoic acid (TNB) to its disulfide, 5-5'dithiobis (2-nitrobenzoic acid) (DTNB) with a resultant decrease in absorbance which can be measured spectrophotometrically. Generation of HOCl is indicated by taurine chloramine formation. TNB was prepared by sodium borohydride (Sigma Chemical Company) reduction of DTNB (Sigma Chemical Company). Reagents were made up on the day of each assay. Isolated PMNs (2.6 x 10^6) were resuspended in phosphate buffered saline (PBS) in the presence of 15 mM taurine and 0.77 mM TNB (pH 7.4) and placed in a

cuvette at a final volume of 2.6 ml. Neutrophils were then activated by the addition of 100 nmol phorbol myristate acetate (PMA, Sigma Chemical Company) and TNB oxidation was followed spectrophotometrically (Shimadzu UV-160) at 412 nm for 20 minutes at 37°C under continuous stirring. Assays were performed in duplicate and a reference cuvette containing cells and reaction mixture which remained unstimulated was used with each assay to adjust for spontaneous oxidation of the indicator. The amount of HOCl generated per minute during the 20 min assay period was calculated from the absorbance change at 412 using an absorption co-efficent of 1.36×10^4 M^{-1} cm^{-1}. Data were expressed as nmol HOCl/ 10^6 PMNs / min. Cumulative production of HOCl was determined for each cell sample from the total optical density change over 20 minutes. Results are expressed as nmol HOCl/ 10^6 PMNs /20 min.

Lung Myeloperoxidase (MPO) Content.

At 300 minutes, animals were sacrificed by infusion of pentobarbital (100 mg/kg) and the right lung immediately excised. One gram samples were obtained and homogenized (Virtis s-45 homogenizer, Virtis, NY) in 4 ml of 20 mM potassium phosphate buffer (pH 7.4). The homogenate was then centrifuged (40,000 g, 4°C, 30 min, Beckman L5-65 Ultracentrifuge, Fullerton, CA). The pelleted material was resuspended in 4 ml 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl ammonium bromide (HTAB)(Sigma Chemical Co. St Louis, MO) and frozen at -70°C. Prior to assay batched samples were thawed, sonicated x 90 sec, incubated for 2 hrs (60°C) and centrifuged (1000 g, 30 min, 4°C). MPO content was assessed by its peroxidation of dimethoxybenzidine (DMB, Sigma, Chemicals). The average change in OD over the period of observation was compared with OD of 10^6 freshly isolated porcine PMNs prepared in an identical fashion as noted for lung parenchymal samples. Results are expressed as units of equivalent MPO activity per gram of lung tissue.

Pulmonary compliance measurements

Pleural pressure was measured by positioning an esophageal balloon (National Catheter) in the mid-esophagus where changes in esophageal pressure were most negative and interference in the pressure signal due to cardiac motion was minimized. Airway pressure was measured from a side port of the ventilator tubing just proximal to the endotracheal tube. Airway and esophageal pressure catheters were connected across a differential pressure transducer (Validyne MP45-4, range + 50 cm H_2O, Validyne Engineering Corp., Northride, CA) for measurement of transpulmonary pressure. Exhaled volume was measured by connecting a pneumotachograph (Model 8805B; Hewlett-Packard, Waltham, MA) in line with the ventilator exhalation tubing which was then coupled to a flow transducer (Model 47304A; Hewlett-Packard, Waltham, MA). The airflow signal was sent to a respirator integrator (Model 8815A; Hewlett-Packard, Waltham, MA) for volume determination. Volume was electronically plotted against both airway and transpulmonary pressures (X-Y Recorder Module; Warren E. Collins Inc., Braintree, MA). Pressure transducers were calibrated daily using a water manometer; the pneumotachograph and volume integrator were calibrated using a precision syringe.

For measurements of dynamic compliance (C_{dyn}), the pneumotachograph was placed at the exhalation port of the Harvard Ventilator for volume determination. A minimum of eight tidal volumes occurred before each dynamic compliance measurement to assure stabilization at functional residual capacity (FRC). Dynamic compliance was determined by the formula:

$$C_{dyn} = \frac{\text{Tidal Volume/Peak Inspiratory Pressure - End Expiratory Pressure}}{\text{Weight in Kg}}$$

Static lung compliance (C_L) was determined by briefly connecting the animal to a pressure ventilator (Bird, Puritan-Bennet Corp.). The pneumotachograph was placed at the distal end of the exhalation tubing in this system. A hand operated,

spring-loaded, on-off valve (Model BE137; Instrument Industries Inc., Bethel Park, PA) was introduced mid-way between the endotracheal tube and the pneumotachograph to halt exhaled airflow at 0.5 sec intervals. Since the exact total lung capacity in these animals was unknown, the lungs were inflated with pressurized 0_2 to 25 cms H_20 pressure and then permitted to deflate passively. Static pressure/volume measurements were obtained by allowing exhalation to occur stepwise until functional residual capacity (FRC) was reached. Intervals of at least 0.5 seconds were inserted between each exhalation burst to ensure that pressures had stabilized. Each compliance measurement included at least eight data points throughout exhalation. Static lung compliance was computed from the line of best fit applied to the pressure-volume curve by the following formula:

$$C_L = \frac{\text{(Change in Lung Volume/Change in Transpulmonary Pressure)}}{\text{Weight in Kg}}$$

Static compliance of the respiratory system, (lung and chest wall combined) commonly measured in intensive care unit patients was not measured in this study. Measurement of C_L is independent of airway and chest wall contributions, and is specific for pulmonary parenchymal elasticity. C_{dyn} was measured to assess whether it would accurately reflect changes in C_L.

Nitrite Assay (Endothelial Derived Relaxing Factor)

Arterial nitrite was measured by a modified method of Green based on the Griess reaction using an automated microplate system (Bio-Tek EL 309) (Green et al.1882). Thirty five percent 5-sulfosalicylic acid (40 µl) was added to plasma samples (200 µl), and the mixture was vortexed for 30 minutes to deplete protein present in samples. After 15 minutes of centrifugation at 10,000 g (Eppendorf 5415, Brinkman), 50 µl of the resulting supernatant was placed into triplicate wells of 96 well flat bottom microplates (Costar, Cambridge, MA). An assay buffer (100 µl) consisting of NH_4Cl

(5%)/ NaOH (5%) (5:2 v:v in distilled water) was added to each well. One hundred µl of the Griess reagent (1% sulfanilamide in 5% phosphoric acid:0.1% N-1-Naphthylethylenediamine dihydrochloride (NEDE)) was then added, and the system was allowed to react under constant agitation for 15 minutes at 22°C. Optical density of samples was determined at 550 nm. A standard curve was generated from known concentration of sodium nitrite in distilled water prepared under similar conditions. Nitrite concentration in unknown samples was calculated by linear regression analysis of a standard curve from 0.04 µM to 6.0 µM with an average coefficient of determination r^2=0.98.

Immunoreactive Endothelin Assay

Immunoreactive Endothelin-1 (ET-1) was determined using a ET 1-23 [^{125}I] assay system based on competition between unlabelled ET-1 and a fixed quantity of [^{125}I] labeled ET-3 (synthetic) for a limited number of binding sites on an ET 1-21 specific antibody (Amersham UK)(Stewart et al.1991). Briefly, plasma was acidified with 250 µl of 2.0 M HCl and centrifuged at 10,000g x 5 min at 4°C. C2 columns (Amersham UK) were equilibrated with 2 ml methanol followed by 2 ml water at flow rate < 5ml/min. Samples were loaded, and the columns were washed with 5 ml of 0.1 trifluoroacetic (TFA) acid (Fisher Scientific, Pittsburgh, PA). Elution was achieved with 2 ml 80% acetonitrile in 0.1% TFA, and the samples were dried in a centrifugal evaporator (SepVac) and reconstituted in 0.02 M borate/0.1% sodium azide buffer pH 7.4. Each sample was prepared in duplicate, and a standard curve from 0.62 to 39.87 pg/tube was used with unknowns calculated by linear regression equation derived from logit B/Bo vs log ET-1 (B=bound radioactivity in presence of standard, Bo=bound radioactivity at zero concentration of ET-1).

The ET 1-23 antibody has 100% cross reactivity to ET-1, ET-2, and 52% to ET-3. It has 0.26% cross reactivity to inactive porcine big endothelin. Interassay variation was 8-10% and intraassay precision was 3-6%. Recovery is known to be 79%.

Pulmonary Intravascular Macrophage Studies

Placement Of In-Situ Pulmonary Perfusion Catheters. Animals were pre-anesthetized with ketamine (25 mg/kg) and restrained supine. General anesthesia was induced by intravenous Pentobarbital (30 mg/kg). Tracheal intubation was performed using a cuffed endotracheal tube (National Catheter) and mechanical ventilation was begun (Harvard Large Animal Ventilator, tidal volume, 10 ml/kg body weight). Sodium heparin (6,000 units, Elkins-Sinn) was administered by intravenous bolus and animals were sacrificed (Pentobarbital 100 mg/kg, IV) 20 minutes later. Mechanical ventilation was continued throughout the perfusion procedure. A median sternotomy was performed and the pericardium opened without entering the pleural spaces. The superior vena cava, inferior vena cava, and ascending thoracic aorta were ligated. The left atrial appendage was opened and a #40 french Sarns catheter (Sarns, inc.) was positioned at the confluence of the pulmonary veins. The catheter was secured in the left atrial appendage using a purse string suture. A 2 cm transmural incision was made in the right ventricle proximal to the pulmonary outflow tract and a #32 french catheter was positioned in the main pulmonary artery proximal to its bifurcation. Perfusion catheters were secured by umbilical tape and sterile tubing to capture pulmonary effluent was attached to the left atrial ccatheter.

Preparation of Perfusion Fluids.

All solutions were prepared using aseptic technique. Reagents were obtained from Sigma (St Louis, MO), dissolved and filter sterilized (0.22 μm) prior to mixing in intravenous infusion bags (Baxter/Travenol) containing sterile NaCl (0.9%). The following solutions were prepared as previously described: (1) 0.01% EDTA, 1 liter; (2) 0.075% sodium nitrate, 500 ml; (3) 0.055% calcium chloride, 1 liter; (4) 0.025% collagenase type IA plus 0.055% calcium chloride, 1 liter.

Harvesting Anchored Mononuclear Phagocytes. Perfusion of the pulmonary vasculature was performed by placing fluid bags 30 inches above the pulmonary artery.

Infusion fluids were administered in the following order: (1) EDTA, 500 ml; (2) $NaNO_3$, 500 ml; (3) EDTA, 500 ml; (4) $CaCl_2$, 500 ml; (5) collagenase/$CaCl_2$, 1000 ml; (6) $CaCl_2$, 500 ml. Pulmonary effluent obtained prior to infusion of collagenase/$CaCl_2$ solution was discarded. Subsequently, effluent was collected into iced, sterile 250 ml centrifuge tubes (Corning).

Isolation Of Cells Retrieved From Lung Perfusion. Lung perfusion effluent was centrifuged (400 g, 4°C, 15 minutes) and resulting cell pellets washed x 2 in phosphate buffered saline without Ca^{++} or Mg^{++} (PBS, GIBCO, Grand Island NY), containing 4% Penicillin/Streptomycin (P/S, GIBCO). Cells were resuspended in PBS containing 1% P/S, layered over a 60% Percoll® cushion (Pharmacia), and centrifuged (1000 g, 22°C, 10 minutes). Following centrifugation, the upper 20% of the gradient was removed and washed in PBS with 1% P/S. Examination of cells in lower regions (i.e., bottom 15%) of the Percoll® gradient revealed mixed cell populations which consisted predominately of neutrophils and lymphocytes with < 5% of cells exhibiting monocyte morphology. The desired cellular fraction was layered over a second gradient Percoll® cushion, centrifuged, and the upper 20% of the gradient removed. Cells were washed twice, resuspended in PBS (1% P/S) and counted manually using a hemacytometer. Viability was determined by Trypan Blue dye exclusion. Differential cell counts were obtained by counting at least 200 cells on modified Wright-Giemsa stained (Dif-Quik®, American Scientific Products, McGaw Park IL) cytocentrifuge preparations (Cytospin 2, Shandon Inc., Pittsburgh, PA).

Cell diameters were measured using a Leitz Orthoplan® light microscope containing an eyepiece reticle. Thirty cells from random fields on Wright-Giemsa stained cytospins were measured at 500X magnification. Results were compared to measurements performed on cytospin preparations of previously isolated alveolar macrophages and blood monocytes. Results are expressed as mean cellular diameter (μm).

Non-Specific Esterase Stain. Non-specific esterase staining was performed after the method of Yam et al. Briefly, cytocentrifuge preparations were fixed in a Coplin jar for 15 seconds in Citrate/Acetone/Formaldehyde solution at 22°C with vigorous agitation for the last 5 seconds of incubation. Slides were rinsed in deionized water for 60 seconds and immediately flooded with a solution containing freshly mixed β-naphthyl acetate, Fast Blue BB Base, Trizmal, and sodium nitrate (Sigma). Slides in this staining solution were incubated in the dark at 37°C for 30 minutes, rinsed with deionized water x 2, counterstained with hematoxylin (2 min), air dried, and preserved under coverslip (Permount). Cells exhibiting blue/black or diffuse tan-brown staining of cytoplasm or cytoplasmic accumulations of brown granules were considered to be non-specific esterase positive. Two hundred cells were graded positive or negative for non-specific esterase staining.

In-Vitro Phagocytosis. Immediately following isolation, cells were adjusted to a concentration of 1×10^6 cells/ml in Dulbecco's modified eagles medium (DMEM), containing 5% calf serum and 1% P/S. Aliquots of cell suspension (300 µl) were added to separate wells of two sterile tissue culture chamber slides (Lab-Tek). Slides were incubated for two hours at 37°C, 5% CO_2 to permit cell adherence. Following incubation, cell wells were gently washed twice with PBS containing cations. Three hundred µl of DMEM with or without particulate carbon suspension (200 x dilution of stock india ink suspension) was added to each cell well on both slides. Slides were incubated for 1 hour at either 4°C or 37°C, were washed x 4 with PBS, air dried, stained with Dif Quik® and preserved under coverslip. Two hundred cells from random fields were counted on slides incubated under both temperature conditions. Cells demonstrating uptake of 2 or more carbon particles were considered phagocytic. Results are expressed as a phagocytic index established by dividing phagocytic cells by the total cells counted.

Experimental Design

Cycloxygenase Inhibition Using Ibuprofen

Introduction

Inhibition of cyclooxygenase metabolites of arachidonic acid, using the reversible inhibitor ibuprofen, has been shown to attenuate both hemodynamic derangements and enhanced alveolar-capillary membrane (ACM) permeability associated with experimental sepsis-induced acute lung injury. These studies suggest that improvements in oxygenation and the reduction in pulmonary arterial hypertension occur primarily as a result of inhibition of the cyclooxygenase metabolite, thromboxane A2. Both thromboxane (Tx) and prostaglandins (PG) contribute to the ventilation/perfusion mis-matching which develops early in the course of ALI and Tx is in part responsible for severe pulmonary arterial hypertension observed in animal models following LPS or bacterial infusion (Hales et al.1981). A second phase of injury in septic animals is characterized by increased ACM permeability resulting from endothelial cell injury (Brigham and Meyrick, 1986; Brigham et al.1974).

Ibuprofen exerts a number of effects on neutrophils *in-vitro*. Nielsen et al demonstrated inhibition of lysosomal enzyme release, and down regulation of PMN superoxide anion (O_2^-) generation (Nielsen and Webster, 1987). Further, *in-vivo* studies of experimentally induced lung injury in primates and rats suggests that ibuprofen reduces PMN mediated tissue injury (Kopolovic et al.1986; Lee et al.1986).

Ibuprofen Pretreatment Studies.

Animals were randomly divided into three study groups. Control animals (n=7) received an infusion of sterile 0.9% NaCl for 60 min. Septic animals (n=8) were infused continuously for 1 hour with live *Pseudomonas aeruginosa* (PAO strain, $5x10^8$ organisms/ml at 0.3ml/20 kg/min). The treatment group (n=6) received an identical infusion of live organisms as well as ibuprofen (12.5 mg/kg infused at 0 and 120 minutes).

Delayed Ibuprofen Treatment Studies.

Study groups included Control (n=5), septic group (n=10) and a delayed treatment ibuprofen group IbuPs (n=9) animals respectively. Ibuprofen delayed treatment animals received an identical one hour infusion of *Pseudomonas* but were given ibuprofen (12.5 mg/kg) 30 minutes into the *Pseudomonas* infusion and then again at 120 minutes. All animals weighed between 15 and 25 kg with a mean weight of 22.1 ± 1.4 kg with no significant differences between the groups.

Combined Cimetidine, Ibuprofen and Diphenhydramine Studies

Again, three groups of animals were studied. Control animals (n=6) received a sham infusion of 0.9% NaCl equal to the volume and rate of the Pseudomonas infusion. The septic group (n=5) was infused continuously with live *Pseudomonas aeruginosa*, ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min) and the treatment group (n=6) received Pseudomonas plus the following pharmacological agents beginning at 90 min post Ps infusion: cimetidine (150 mg) and ibuprofen (12.5 mg/kg), given hourly by bolus intravenous infusion, and diphenhydramine (10 mg/kg/hr) given in a continuous intravenous infusion diluted in 100cc normal saline. Diphenhydramine was administered as an infusion to avoid the hypotensive effects associated with bolus injection.

CD18 Adhesion Receptors

Introduction

Experimental models of acute lung injury produced by infusion of live bacteria or bacterial endotoxin (lipopolysacharide, [LPS]) are associated with a prompt and significant reduction of circulating neutrophils (PMNs). The pathogenesis of neutropenia and the mechanisms responsible for neutrophil sequestration in the lung in sepsis remain incompletely defined. Sequestration of activated PMNs in the pulmonary microvasculature may result from increased adhesion to pulmonary endothelium (Harlan et al.1985). The CD11/CD18 glycoprotein adhesion complex on the PMN cell surface facilitates augmented adhesion of cytokine or LPS activated PMNs to vascular endothelium in vitro (Harlan, 1987; Vedder et al.1989). *In vitro* adhesion of LPS activated porcine PMNs to porcine pulmonary endothelium is CD18-dependent with CD11/CD18 adhesion receptors producing arrest of activated PMNs in pulmonary capillaries *in vivo* (Larson and Springer, 1990; Carlos and Harlan, 1990). Thus, there is evidence to suggest that CD11/CD18 is involved in "trapping" activated PMNs in the pulmonary microvasculature.

Monoclonal antibody 60.3 (MoAb 60.3) is a murine IgG2a monoclonal antibody that recognizes a functional epitope on the CD18 subunit of the CD11/CD18 adhesion complex. It is of proven benefit in attenuating PMN induced reperfusion injury in experimental hemorrhagic shock (Vedder et al.1988).

Initial studies were carried out to examine the expression of CD18 receptors on porcine neutrophils both in vitro following TNFα stimulation and in vivo *Pseudomonas* stimulation. Following these studies the monoclonal antibody 60.3 used in the above studies as the primary fluorescent stain, was also utilized as a treatment agent in an attempt to prevent neutrophil mediated tissue injury. MoAb 60.3 was used in this study to examine the hypothesis that neutropenia and pulmonary PMN sequestration, with subsequent transendothelial migration into the alveolar space and local tissue injury,

are CD18 dependent phenomena in sepsis.

Control animals (n=8) received an infusion of sterile NaCl (0.9%) for 60 min. Septic animals (n=9) were infused for 60 min with live *Pseudomonas aeruginosa* PAO strain ($5x10^8$ organisms/ml at 0.3ml/20 kg/min). A third group of animals (n=7) were pretreated with monoclonal antibody 60.3 (MoAb 60.3) prior to Pseudomonas infusion. Fifteen minutes following MoAb 60.3 infusion the 1 hr infusion of *Pseudomonas aeruginosa* was commenced.

Purified MoAb 60.3 was provided (Bristol Myers Squibb, Oncogen Division, Seattle, WA.) at a concentration of 2.6 mg/ml in 50 mM sodium phosphate buffer, pH 7.0. Samples were stored at -70°C after aliquoting to avoid freeze thaw cycles. Prior to each experiment MoAb 60.3 was thawed and reconstituted in 25 ml sterile saline. The antibody (2mg/kg) was infused over 15 min via the left internal jugular vein. Serum antibody concentration was measured by an enzyme linked immunosorbent assay (ELISA) immediately following infusion and again at 180 and 300 min.

Cytokine Studies

Introduction

Cytokines play an important role in inflammation, both by direct action on cells at sites of infection and by trafficking other cells of the immune system, such as polymorphonuclear phagocytes (PMNs) (Mantovani and Dejana, 1989). Of the numerous cytokines now recognized, tumor necrosis factor-α (TNFα) has emerged in recent years as a critical chemical mediator of sepsis syndrome (Rock and Lowry, 1991). TNFα is a 17 Kd peptide produced predominantly by members of the mononuclear phagocyte system in response to particulate and soluble inflammatory stimuli (Rock and Lowry, 1991). As discussed above, a growing body of evidence implicates blood PMNs as primary mediators of end organ damage associated with multisystem organ failure in sepsis, particularly the alveolar capillary membrane damage characteristic of sepsis-associated acute lung injury (Tate and Repine, 1983). Using the porcine model of septic acute lung injury, this laboratory has correlated the appearance of TNFα in the circulation with priming of PMNs for toxic oxygen metabolite generation, increased expression of PMN $ß_2$ integrins and consequent loss of PMNs from the circulation (vide infra) (Walsh et al.1991). *In vitro* studies indicate that TNFα activates PMNs for oxidant generation, phagocytosis, degranulation and adherence (Larrick et al.1987). Though augmentation of these critical functions primarily prepares PMNs for first line immune defense, these functions are equally capable of precipitating host tissue damage.

Pretreatment Anti-TNFα Monoclonal Antibody Studies

This study examined the effects of anti-TNFα MoAb pretreatment on the course of gram negative sepsis and acute lung injury produced by infusion of live *Pseudomonas aeruginosa*. The IgG1 anti-TNFα monoclonal antibody (anti-TNFα MoAb) used in this study was provided by Cutter Biological, Miles Inc. Berkley CA. The antibody was purified from murine hybridoma culture harvests via cell separation,

polyethylene glycol precipitation, anion exchange and size exclusion chromatography. Purified MoAb was 99% pure with fully functional binding to human TNFα. Endotoxin levels were less than 2 U/mg protein (Limulus assay). Stabilization was performed with glycine and maltose prior to lyophilization. Lyophylized MoAb was kept at 4°C until time of experiment, at which stage it was reconstituted with 20 mls of sterile water. Anti-TNFα MoAb (15mg/kg) was administered over 10 min via the left internal jugular vein.

Three groups of animals were studied. Two groups received a 1 hour infusion of live *Pseudomonas aeruginosa* ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). One of these groups was pretreated with intravenous monoclonal antibody against TNFα (anti-TNFα MoAb, 5 mg/kg) 15 min prior to commencing Pseudomonas infusion. The remaining group received sterile saline only. All animals were studied for 300 min.

Combined Anti-TNFα and Ibuprofen Blockade

Three groups of animals were studied. Septic and treatment animals received a 1 hour infusion of live *Pseudomonas aeruginosa* ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). Treatment animals also received anti-TNFα, 5 mg/kg and ibuprofen 12.5 mg/kg, 15 minutes prior to the bacterial infusion and a further bolus of ibuprofen alone at 120 minutes into the study. Control animals received sterile saline only. All animals were studied for 300 min.

Post Treatment Anti-TNFα Blockade

Once again, three groups of animals were studied. Control, received a 60 minute intravenous infusion of sterile saline. Sepsis, received a 60 minute intravenous infusion of live *Pseudomonas aeruginosa*, PAO strain ($5x10^8$ CFU/ml at 0.3 ml/20 kg/min). Anti-TNFα treated animals received a 60 minute infusion of live *Pseudomonas aeruginosa*, following which they received a bolus infusion of monoclonal antibody to TNFα (5 mg/kg I.V.).

Miscellaneous

Soluble Complement Receptor (sCr1)

Complement depletion in a porcine model using cobra venom factor has been shown to exert a protective effect on the alveolar-capillary membrane as evidenced by reduced extra vascular lung water (Dehring et al.1987). In addition the rise in pulmonary artery pressure and fall in arterial pO_2 were also attenuated. Complement receptor blockade represents another way of studying the effects of complement inactivation in animal models of acute lung injury.

The activation of C3 and C5 constitute a critical step in the activation pathway of complement and accordingly is tightly regulated *in vivo*. Of the five proteins that inhibit the activation of C3 and C5, two are in plasma and the remaining three are membrane bound. Of these latter three proteins, one is called complement receptor 1 (CR1) and is able to act as a highly specific inhibitor of complement activation through both the classical and alternative pathways. The distribution of this receptor, however, is relatively limited, being found primarily on erythrocytes and leukocytes. This drawback has been overcome by synthesis of a truncated form of CR 1 (sCr1), lacking the transmembrane and cytoplasmic domains of the molecule. With these modifications, it is highly soluble and capable of suppressing complement *in vivo* and has been shown to reduce inflammatory tissue damage in a number of experimental settings (Fearon, 1991; Weisman et al.1990).

The current study was performed in order to ascertain whether selective blockade of the complement cascade utilizing sCRI is capable of attenuating the acute lung injury induced by Pseudomonas infusion in a porcine model.

Three groups of animals were studied, control and septic as previously described and an sCr1 treatment group. The sCr1, was administered in equally divided dosages prior to and following induction of sepsis. Three animals were given 15 mg/kg and the remaining two animals were given 30 mg/kg.

Nitric Oxide Levels in Sepsis

TNFα and endotoxin promote release from vascular endothelium of endothelium-derived relaxing factor (EDRF) and endothelin (ET), both potent mediators of vascular tone (Salvemini et al.1990; Moncada et al.1991; Yanagisawa et al.1988). EDRF is now characterized as nitric oxide, and is produced in response to inflammatory and physical stimuli such as increased shear forces as occurs with enhanced blood flow during sepsis (Moncada et al.1991). Endothelin is an endothelium derived peptide that produces sustained vascular contraction in porcine and human vasculature (Salvemini et al.1990). Kilbourne provided additional evidence linking TNFα to endothelial production of vasoactive mediators by showing that TNFα induced hypotension could be reversed by infusion of the EDRF antagonist, N^G-monomethyl-l-arginine (LNMMA) (Kilbourn et al.1990). EDRF represents but a single agent by which vascular endothelium regulates vessel tone. Both EDRF and endothelin can modulate other potent vasopressors which regulate vessel tone. Thus, regulation of EDRF and ET production is complex.

Refractory septic shock is characterized by lack of efficacy of catecholamines (e.g. norepinephrine, phenylephrine, and dopamine) as a therapeutic maneuver to moderate hypotension. This phenomenon may be due in part to excessive release of EDRF (Julou Schaeffer et al.1990). In view of the multiple interactions between EDRF and ET, it has become clear that both mediators must be studied simultaneously during a septic process.

In the complex paradigm of sepsis, vascular endothelium emerges as a regulator of immunological function and also serves as a rheostatic organ which modulates vasomotor tone. This study examined circulating levels of nitrite (as a marker of EDRF release) and immunoreactive endothelin levels, during the course of gram negative septic shock and acute lung injury in pigs and, investigated the effect of pretreatment with the monoclonal antibody to TNFα . Three groups of animals were studied. The

control group consisted of 5 animals. The septic group contained 7, and the antibody treated group (pretreatment with anti-TNFα monoclonal antibody 5 mg/Kg) contained 7 animals.

TGF-ß$_1$ and Pulmonary Intravascular Macrophage (PIM) Studies

Mononuclear phagocytes are known to play a central role in lung repair through release of various cytokines and growth factors. Pulmonary intravascular macrophages (PIMs) may modulate repair mechanisms following septic lung injury from within the pulmonary capillaries. We hypothesized that PIMs influence the course of angiogenesis and repair of the interalveolar septum following septic acute lung injury through local production of growth factors.

In order to test this hypothesis, we set out to demonstrate that PIMs are capable of producing TGF-ß$_1$ mRNA, that mRNA for TGF-ß$_1$ increases following exposure to LPS and finally to assess the effect of coincubation with TGF-ß$_1$ and other cytokines on PIM mRNA production.

PIMs were harvested as previously described and the effluent centrifuged having been layered over 60% isosmotic Percoll. The cells at the interface were then collected, counted and checked for viability. Following culture with serum-free media and incubation with LPS, IL-1 and TGF-ß$_1$ for a 24 hour period, the cells were lysed and RNA extracted. Northern Blot analysis was performed with a[^{32}P]-labelled TGF-ß$_1$ cDNA probe

Results

Ibuprofen Studies

Physiological measurements.

Pretreatment ibuprofen

Animals in the septic group exhibited rapid onset of pulmonary arterial hypertension, peaking at 46 ± 1.6 mmHg within 30 minutes of commencing infusion of live Pseudomonas. Pulmonary pressures moderated following the peak at 30 min but remained significantly elevated above control for the duration of the study ($p<0.05$). Infusion of ibuprofen into septic animals resulted in a significant attenuation of early onset pulmonary artery hypertension. Subsequently a gradual rise in PAP occurred resulting in levels equivalent to those in septic animals at 240 and 300 min. Systemic arterial pressure in septic animals fell significantly below controls by 120 minutes with significant hypotension persisting to 300 minutes. Ibuprofen administered to septic animals maintained SAP at or above control levels throughout the study. In septic animals PaO_2 fell below control levels shortly after outset of the study becoming significantly depressed at 30 minutes. No changes in PaO_2 from baseline was observed in controls. Infusion of ibuprofen into septic animals maintained PaO_2 at control levels until 240 and 300 min. Cardiac index fell progressively in septic animals with significant depression compared to control evident at 30 minutes and from 180 minutes onwards. Cardiac index in the treated animals was indistinguishable from septic group for the duration of the study. As previously documented in this septic porcine model PAOP remained at baseline levels in all groups until 300 min.

Delayed Treatment Ibuprofen.

Delayed infusion of ibuprofen resulted in a significant reduction in PAP from a 30 minute peak of 47±3 mmHg to 16±1 mmHg at 60 minutes. Subsequently PAP gradually rose and became significantly different from control at 180 240 and 300 minutes. Delayed ibuprofen treatment maintained SAP significantly above septic unprotected

values for the entire study and, prevented the fall in PaO_2 observed in septic animals. In contrast the biphasic decline in cardiac function typical of unprotected sepsis was still observed in the ibuprofen treated group.

Delayed, Combined Cimetidine, Ibuprofen and Diphenhydramine (CID) Studies

SAP remained significantly higher in the CID group compared to the septic group, achieving statistical significance at 120 min. SAP (Figure 1) was significantly lower in the CID group compared to control animals from 180 min until the end of the study. PAP (Figure 2) in the septic group was increased at 30 min, and remained significantly higher than control animals throughout the study. This effect was unaltered by CID therapy. Cardiac Index (Figure 3) in septic animals showed progressive deterioration becoming significantly lower than baseline at 120 min. Treatment with CID maintained Cardiac Index at control levels until 240 min and significantly higher than at 180 and 210 min. Arterial oxygen tension (Figure 4) in septic animals showed a progressive deterioration becoming significantly lower than control animals at 90 min. Treatment with CID at 90 min delayed further decline after 120 min, maintaining PaO2 at levels significantly septic untreated above at 180 and 210 min, though significantly lower than control animals from 120 to 300 min. This study, unlike the others addressed survival, treatment with CID significantly prolonged animal survival compared to septic animals ($p < 0.01$). The mean survival time in the septic group was 199 ± 34 min. The mean survival time in the CID group, 328 ± 45 min, was significantly ($p < 0.01$) longer. At 270 minutes post infusion, all 6 animals CID treated animals were still alive compared to none in the Ps alone group.

Oxidant Generation

Figure 5 shows the results of total PMN superoxide generation (10 min assay period) from control, septic and ibuprofen treated animals at 0 and 60 minutes. This figure shows no change in superoxide radical generation from control PMNs at these time points. This suggests that experimental manipulation, surgery and prolonged

ventilation using high inspired O_2 concentrations did not effect PMN oxidant production. In contrast, PMNs from animals which received an infusion of live Pseudomonas organisms demonstrated marked enhancement of oxidant production as evidenced by a significantly increased total superoxide radical production (Figure 5) and a more acute kinetic rise to a greater peak production noted in figure 6. This suggests that cells which remain in the peripheral blood after the onset of a profound bacteremia, as is the case in this model, can mount an effective antibacterial challenge and are "primed" to produce oxidants in greater quantities.

Addition of ibuprofen, either as pre or posttreatment, to septic animals resulted in a dramatic reversal of superoxide anion generation upregulation. As can be seen from both figures generation of superoxide anion in ibuprofen treated animals parallels that of the control group. Interestingly, treatment with CID therapy caused a reversal of this attenuation, where CID treated cells were then able to produce superoxide radical levels equivalent to those of cells from septic unprotected animals.

Hypochlorous Acid Production

In control animals, generation of taurine chloramine (HOCl) reached peak production at 8-10 minutes following PMA stimulation. No differences in PMA stimulated HOCl production by neutrophils obtained at 0 or 300 min was observed (1.18 ± 0.08 vs 1.2 ± 0.1 nmol/10^6 PMN, $p=0.29$ ANOVA) in control animals. In contrast, PMNs from septic animals exhibited greater rates ($p<0.05$) of HOCl production for up to 14 minutes following PMA stimulation when compared to PMN obtained at baseline in the same animals. A 25% increase in maximum HOCl production (1.7 nmol/10^6 PMN) occurred 10 minutes following PMA stimulation in septic PMNs ($p<0.01$).

Effect of ibuprofen on hypochlorous acid production.

Despite significantly increased rates of HOCl production in the first 5-6 minutes following PMA stimulation, the presence of ibuprofen in septic animals resulted in a significant decrease in oxidant generating capacity at all assay timepoints when

compared to septic PMN alone ($p<0.05$). At 20 minutes following PMA stimulation, PMN from septic animals obtained at 300 minutes generated significantly greater quantities of HOCl, 9.84 ±0.4, 0 min vs 13.02 ± 0.3, 300 min nmol/10^6 PMNs ($p<0.01$, Students t-test) than all other groups. This quantitative increase in HOCl production was not detected in PMNs from Ps infused animals at 60 minutes. The quantitative increase in long-lived oxidant generation at 300 min in septic animals was 32% higher when compared to PMN obtained at baseline. Pretreatment and post treatment of animals with ibuprofen attenuated HOCl production from PMN obtained at 300 minutes and this was indistinguishable from that observed in control animals (9.79 ± 0.4 vs 10.12 ± 0.1 nmol/10^6).

Compliance Studies

Dynamic compliance (C_{dyn}) remained at or near baseline levels throughout the study in control animals. In septic animals however, C_{dyn} decreased to 73% and 67% of baseline values at 30 and 60 minutes following the onset of Pseudomonas infusion. Values in Pseudomonas infused animals decreased to 36% - 45% of baseline from 150 mins to the end of the study. C_{dyn} in septic animals became significantly decreased compared to controls ($p<0.01$) at 30 mins and remained significantly lower throughout the study (Figure 7).

Static lung compliance (C_L) remained at or near baseline levels throughout the study in controls. In septic animals, C_L decreased to 61% and 54% of baseline values at 30 and 60 minutes respectively. Values thereafter remained between 40% - 57% of baseline. Static compliance measurements in septic animals were significantly lower than controls from 30 mins until the end of the study ($p<0.01$). Ibuprofen maintained C_L at or near control levels throughout the study, significantly higher than the septic group, and also CID treatment caused significant attenuation of the fall of pulmonary compliance (Figure 8).

Alveolar capillary membrane permeability

Extravascular lung water measurements in the control animals remained at baseline levels throughout the study. A sustained rise in EVLW in septic animals was observed. Statistical significance became apparent at 120 minutes, reaching a maximum of 16.2±1.7 ml by 300 min ($P<0.05$ vs Con, 120-300 min). Ibuprofen pretreated animals exhibited a gradual rise in EVLW, reaching a peak of 10.1±1.2 ml at 180 min with a subsequent fall to 8.9±1.0 at 300 min. Extravascular lung water in treated animals was significantly less than that observed in septic animals at 240 and 300 min. At no time were EVLW levels significantly elevated above control values in Ibuprofen group ($p >0.05$ 0-300 min). Delayed treatment similary improved the accumulation of extra vascular lung water seen in the septic unprotected animals, however this protection appeared to be lost by the addition of cimetidine and dyphenhydramine.

Bronchoalveolar Lavage Protein Content (BAL-P)

Bronchoalveolar lavage returns for the described procedure were consistently high 75-79% in all animals in this study. A significant increase in BAL protein content was observed in septic animals at 300 minutes. BAL protein content in septic lavage was 5.2 times that observed at baseline in the same group (0min, 130±22 µg/ml vs 300 min, 683±133 µg/ml, $p<0.01$). In contrast, there was no protein accumulation in the airspace over 5 hrs in control animals. Pretreatment and delayed ibuprofen treatment in septic animals prevented accumulation of protein at the 300 min timepoint, thus attenuating the alveolar capillary permeability injury (Figure 9).

Circulating PMN Counts

Baseline peripheral neutrophil counts for all animals in this study was 23.4 ± 1.6 x 10^3 cells/µl (range 17.0 - 31.5 x 10^3 cells/µl). Total PMN count remained at baseline levels throughout the 300 minutes of study in control animals. In septic animals, total circulating PMN counts dropped soon after the *Pseudomonas* infusion. Significant

neutropenia was well established within 30 minutes and persisted to the end of the study. Similarly, septic animals preinfused with ibuprofen and delayed treated animals, exhibited rapid onset neutropenia parallelling that in septic unprotected animals (Figure 10).

Neutrophil Migration

Assessment of percent PMNs in the alveolar space demonstrated that all groups had minimal presence of PMNs in the airspace under normal conditions. Histologically the neutrophilic alveolitis evident in septic animals at 300 min (24%) was abolished in ibuprofen pretreated and delayed treated groups.

Cytokine Production: The effect of Ibuprofen

Plasma TNFα levels surged in Septic animals within 60 min, reaching a peak of 4.54 ± 0.47 U/ml at 120 minutes, remaining significantly elevated over baseline and control values at 300 min. Control animals showed no significant increases in plasma TNFα activity throughout the study period. Ibuprofen treated animals exhibited increased TNFα levels during the study period, but these levels were significantly lower than those seen in septic unprotected animals (Figure 11).

Adhesion Receptor Studies

In vivo neutrophil CD18 expression

Septic animals exhibited a significant increase in PMN CD18 expression by 60 min ($p < 0.05$ ANOVA). Increased PMN CD18 expression was most marked at 120 min and remained significantly elevated throughout the study. No significant change from baseline in CD18 expression by circulating PMNs was observed in controls (Figure 12).

In vitro neutrophil CD18 expression

Porcine PMNs responded to *in vitro* TNFα stimulation with increased CD18 receptor expression in a concentration and time-dependent fashion. Neutrophils exposed to final concentrations of 10 U/ml and 10^3 U/ml exhibited a significant ($p<.01$) increase in CD18 expression compared to control medium. Time course experiments using TNFα at a final concentration of 10^3 U/ml revealed significantly ($p<.01$) increased CD18 expression by 20 min. Extending the length of incubation to 40 and 60 min maintained this upregulation but yielded no further increases in CD18 expression.

In vivo Effects of Monoclonal Antibody 60.3

Following IV infusion, the serum concentration of MoAb 60.3 was 3.26 ± 0.09 µg/ml. Serum concentrations remained constant and there was no significant change from the post infusion levels over the 300 min study period (Figure 13). The activity of MoAb 60.3 in the serum also remained constant over time.

Hemodynamic Measurements

Prior to the onset of sepsis in the MoAb 60.3 treated group, hemodynamic parameters were measured before and after infusion of the antibody. There was no significant effect of MoAb 60.3 infusion on PAP, SAP, Cardiac Index or arterial pO_2 prior to commencing the Pseudomonas infusion. After the onset of sepsis, MoAb 60.3 treated animals developed identical hemodynamic derangements as untreated animals, namely pulmonary arterial hypertension, systemic hypotension, decreased cardiac index and relative arterial hypoxemia. These changes, not seen in the control animals,

are typical of this model and have been described in detail previously (Lee et al.1986).

Extravascular Lung Water (EVLW)

EVLW was maintained at baseline levels in control animals but was significantly elevated by 180 min in the septic group (Figure 14). There was no increase in EVLW from baseline in the MoAb 60.3 treated animals until the final hour of the study at which time a non significant upward trend was observed when compared with baseline or with 300 min values in control animals.

Bronchoalveolar Lavage

BAL protein concentration was significantly elevated after 300 min of sepsis (1059 ±216µg/ml vs 222 ± 42µg/ml at baseline) (Fig 15). Control animals showed no significant change in BAL protein concentration. Compared with the septic group, pretreatment with MoAb 60.3 caused a significant reduction in BAL protein concentration at 300 min (388 ±75µg/ml). However, this represented a significant elevation compared with baseline (141 ±21µg/ml) within the MoAb 60.3 group, i.e. there was a significant, albeit markedly attenuated, increase in BAL protein. The number of PMNs migrating into the alveolar space (percent PMNs in BAL fluid) at 300 min was unchanged from baseline in control animals but was significantly elevated in Pseudomonas infused animals (29 ± 8% vs 7.3 ± 3% at baseline). MoAb 60.3 reduced PMN migration at 300 min by nearly half (16 ± 4%) but this value failed to reach statistical significance ($p>.05$). The increase in percent PMN concentration over 300 min within the MoAb 60.3 treatment group was not significant (Figure 16).

Blood Neutrophil Count

Control animals maintained a stable peripheral PMN count throughout the 300 min study period (Figure 17). There was an upward trend in the last 3 hours though this was not significant. In contrast, the Pseudomonas group exhibited marked neutropenia which occurred predominantly within the first hour of the study (5.1 ± 0.5 $x10^3$ cells/µl at 60 min vs 24.4 ± 2.0 $x10^3$ cells/µl at baseline). Neutropenia persisted

in septic animals and there was no significant change from the 60 min values over the next 4 hr of the study. In MoAb 60.3 pretreated animals there was a non significant upward trend in PMN count over the first 30 min (27.6 ± 5.6 $x10^3$ cells/μl vs 23.6 ± 2.7 $x10^3$ cells/μl at baseline), followed by a dramatic decrease over the next 30 min (14.6 ± 1.0 $x10^3$ cells/μl at 60 min). However the PMN count was still significantly higher than that observed in septic animals at 60 min ($p<.01$). There was no further fall in PMN count in the MoAb 60.3 group, and PMN counts stayed within 20% of the 60 min values, being significantly higher ($p<.01$) than septic animals at all time points. From 180 min onwards the PMN counts in this group were also significantly lower ($p<.05$) than in controls.

Pulmonary intravascular macrophage studies

Cell Yields Relative to Collagenase Concentration

Twenty-six pigs were studied by the technique described. Of the collectable 1.5 L volume, 924 ± 84 ml were recovered following in-situ lung perfusion. Preliminary studies to determine the optimum collagenase concentration for cell recovery were performed by varying the collagenase between 0.01 and 0.1%. Infusion of high concentrations of collagenase (0.1%) rapidly produced visible disruption of intact lung with accompanying edema and resulted in diminished flow of left atrial effluent (556 ± 40 ml, n=4). Infusion of reduced collagenase concentrations (i.e., 0.01% - 0.035%, n=20) preserved effluent flow (1122 ± 124 ml) with little visible lung disruption. No correlation between perfusate collagenase concentration and cell yield was observed, ($r = 0.3$, NS). Mean total cell numbers obtained in this study was $3.8 \times 10^8 \pm 0.5 \times 10^8$ cells. Cell viability was consistently high (> 98%) and was not altered by changes in collagenase concentration . After preliminary experiments all further animals were perfused at 0.025% collagenase concentration.

Morphological And Histochemical Characteristics Of Retrieved Cells

Heterogenous mononuclear cells populations were obtained from the microcirculation of porcine lung. Most of the cells (71 ± 1%) were small mononuclear cells with indented nuclei and reduced cytoplasmic volume (diameter 10.4 ± 0.27 μm). Larger mononuclear cells with irregular, asymmetric nuclei comprised 25 ± 1% (diameter 16.5 ± 0.31 μm) ($p<0.05$, large vs small mononuclear cells) of observed cells while lymphocytes and neutrophils were 1% and 2% respectively. Porcine alveolar macrophages and blood monocytes exhibited mean cellular diameters of 21.4 ± 0.5 μm and 10.5 ± 0.22 μm respectively. Cellular morphology of cell populations obtained were unaffected by the concentration of collagenase employed in the isolation procedure. Greater than 87% of isolated cells adhered to tissue culture plastic following a 1 hour incubation at 37°C. Morphology and relative composition of 1 hour

adherent cells remained constant compared to initial cell isolates. When allowed to remain in culture for 7 days (DMEM, 5% calf serum, 37°C, 5% CO_2) cells exhibited increases in cell size and cytoplasmic volume. Cells isolated by the technique re-established intercellular adhesion plaques on explanted endothelial surfaces.

Esterase positive staining was observed in 73% ± 5% of cells (n=4). However, staining characteristics differed somewhat from that observed in porcine alveolar macrophages. Both large and small cell populations exhibited diffuse uptake of β-Naphthyl Acetate resulting in homogenous tan/brown staining of cells. Many cells exhibited stippling throughout with fine esterase positive granules. Plasma membrane esterase activity detectable as black "rim like" staining typical of the monocyte/macrophage lineage was identified in both cell populations. In contrast, alveolar macrophages exhibited heavier staining concentrated in coalesced granular areas. Porcine blood monocytes exhibited similar though less intense staining to that observed in mononuclear phagocytes obtained by lung perfusion. Lymphocytes and neutrophils identified on cytospin preparations failed to take up the non-specific esterase stain.

<u>In-vitro Phagocytosis</u>

Mononuclear cells obtained by this technique phagocytosed particulate carbon in-vitro and exhibited a phagocytic index of 80 ± 6 % at 37°C. Cells exposed to identical particulate loads at 4°C exhibited a phagocytic index of 9.5 ± 4 % ($P < 0.01$). No evidence of particle uptake was observed in cells incubated without particulate carbon. Both large and small constituent members of harvested cell populations phagocytosed particulate carbon.

TGF-β_1 And Pulmonary Intravascular Macrophages

These experiments showed that endotoxin (LPS) induced a four fold increase in steady state TGF-β_1 mRNA levels and that this response was maximal after two hours (Figure 18 & 19). RNA production had returned to baseline levels by 24 hours. TGF-β_1 itself induced a 5-8 fold increase in TGF-β_1 mRNA levels produced by PIMs and these were synergistic in increasing mRNA production by PIMs. These experiments suggest that PIMs are indeed capable of expressing the TGF-β_1 gene and that exposure of these cells to endotoxin increases steady state production of TGF-β_1 protein by these cells. In addition we have also shown that TGF-β_1 exerts an autocrine effect on it's own secretion by PIMs. These results support the hypothesis that PIMs may act to modulate lung repair mechanisms and that PIMs themselves are influenced by cytokine networks activated by acute lung injury.

Cytokine Studies

Tumor Necrosis Factor

No significant TNF activity was detected in control pig plasma over 5 hours observation under anesthesia. In septic pigs, TNF activity appeared in plasma 15 mins after onset of sepsis and remained elevated throughout the study period. The appearance of systemic arterial hypotension, declining cardiac output, increased lung water and deteriorating gas exchange correlated with the rise in plasma TNF activity which peaked at 90-120 mins in septic pigs. Our results provide evidence that both TNFα and TNFß are present in plasma in sepsis. Antibodies to TNFα and TNFß neutralized TNF activity in whole septic plasma at 15, 30 and 45 min following onset of septicemia and the antibodies blocked TNF activity in serially diluted septic plasma at all time points up to 210 min of sepsis. TNF activity was not neutralized entirely by TNFα antisera from 210-300 min in septic plasma. These results suggest that TNFα, TNFß and other "TNFα like" activity increases in plasma during septicemia and that this elevated TNFα activity correlates with the development of acute lung injury in the pig.

Pretreatment Monoclonal Anti-TNFα Studies:

Hemodynamic Measurements

Anti-TNFα MoAb significantly improved the declining cardiac index observed in septic animals (Table 4). In these studies animals in both septic groups (antibody treated and untreated) exhibited significant reduction in Cardiac Index over the first 60 min, coinciding with the infusion of Pseudomonas. However, after 60 min, Cardiac Index continued to decline in septic untreated animals (1.3 ± 0.2 L/min/m^2 at 300 min), while a significant ($p<0.05$) recovery of Cardiac Index was observed in antibody treated septic animals following cessation of the Pseudomonas infusion (3.3 ± 0.4 L/min/m^2 at 300 min). From 180 min onward, Cardiac Index in septic animals was significantly lower than that observed in saline controls. Antibody treated septic animals exhibited

no significant differences in Cardiac Index when compared to saline controls from 180 min onward.

Systemic arterial hypertension occurred in the first 30 min following onset of Pseudomonas infusion in both septic groups. In septic, untreated animals, systemic arterial hypertension was followed rapidly by hypotension reaching a nadir at 120 min (82 ± 6 mm Hg). From 120 min onwards, a trend towards recovery of mean systemic arterial pressure was observed. Anti-TNFα MoAb prevented systemic arterial hypotension observed in the untreated septic group (Table 4).

Septic animals developed acute pulmonary arterial hypertension within 30 min of commencing Pseudomonas infusion (Table 4). Early phase pulmonary arterial hypertension was not attenuated by pretreatment with anti-TNFα MoAb. However, pulmonary artery pressures were significantly lower at the end of the study in antibody treated animals compared to septic untreated animals.

Blood Gas Measurement

Septic animals developed an acute and progressive decline in arterial oxygen tension throughout the study, being significantly worse than those observed in the control animals from 60 minutes and beyond. An early fall in arterial oxygen tension was also observed in anti-TNFα treated animals, but from 120 minutes and beyond arterial oxygen tension stabilized and recovered towards the completion of the study period (Figure 20). We observed a gradual 20% decline in mixed venous oxygen tension (PvO_2) over the course of the study in Pseudomonas infused animals. Anti-TNFα treated animals exhibited a rapid 20% drop in PvO_2 during the Pseudomonas infusion; however, this was followed by recovery, such that PvO_2 returned to baseline levels by the end of the study.

Porcine sepsis was invariably characterized by the appearance of a progressive, unremitting metabolic acidosis which intensified toward the end of the study period (arterial pH = 7.16 ± 0.03 at 300 min in the untreated septic group). Anti-TNFα

antibody failed to prevent the appearance of metabolic acidosis during Pseudomonas infusion (0-60 min). However, from 60 min onward, antibody infused animals exhibited no further intensification of the acidosis (Figure 20). At 300 min, arterial pH in group II was 7.39 ± 0.01 and was not significantly different from saline control animals.

Tumor Necrosis Factor and Thromboxane B_2 Activity

Infusion of Pseudomonas produced significant and rapid increases in plasma TNFα levels (Figure 21). Plasma TNFα activity was elevated at 30 min in septic animals and was significantly greater than baseline by 120 min (4.8 ± 0.7 U/ml vs 0.4 ± 0.2 U/ml). Plasma TNFα levels declined thereafter but remained significantly greater than baseline until 240 min. Pretreatment with anti-TNFα MoAb abolished the surge of TNFα observed in the plasma of septic animals with plasma levels exhibiting no significant difference from baseline or saline control animals.

Plasma thromboxane (TxB_2) levels increased sharply following the onset of Pseudomonas infusion and peaked at 30 min (2877 ± 283 pg/ml). TxB_2 levels remained significantly elevated, compared to baseline, throughout the study in septic animals (Figure 22). Pretreatment with anti-TNFα MoAb failed to attenuate the initial increase in plasma TxB_2. Plasma TxB_2 levels in antibody treated animals equalled those observed in septic animals at 30 min and continued to rise until 60 min. Plasma TxB_2 levels subsequently declined in antibody treated animals and were not significantly different from baseline values at 300 min. Saline control animals exhibited no increase in plasma TxB_2.

Peripheral Neutrophil Count

Control animals maintained a stable neutrophil count throughout the entire study period. In septic untreated animals, a profound neutropenia was observed which developed in the first hour of the study and persisted to the end of the study at 300 mins. Septic antibody treated animals however, did not develop a neutropenia and cell counts remained at normal or near normal levels throughout the study period (Figure

23).

Neutrophil CD11/CD18 Expression

PMNs obtained from septic animals exhibited significant upregulation of CD11/18 expression compared to baseline and control values (Figure 24). Peak values were observed from 120 to 240 min. In contrast, PMNs from control and pretreated animals showed no significant upregulation of CD11/18 expression over the course of study.

Lung Neutrophil Load

Myeloperoxidase content of lung tissue from animals in each study group was analyzed to assess lung PMN burden (Figure 25). Septic animals exhibited significantly higher myeloperxoidase content in lung tissue when compared to control animals (51.6 ± 9.9 vs 11.3 ± 2.8 U/g, $p < 0.001$). In pretreated animals, anti-TNFα antibody significantly reduced lung PMN burden when compared to septic untreated animals (25.4 ± 3.3 vs 51.6 ± 9.9 U/g, $p < 0.05$). Thus, antibody treatment reduced lung PMN content despite an ongoing septic process.

Neutrophil Transendothelial Migration

PMN counts in recovered BAL lavage fluid, expressed as a percentage of the total recovered white cell count, were not significantly different between groups at time 0. In septic animals, lavage recovered significantly ($p <0.05$) more PMNs at 300 min (24.5 ±6.7 $p<0.05$) than at 0 min (1.8 ± 0.4) and control animals, at 300 min (3.9 ± 1.4). There was no significant increase in PMNs recovered from BAL at time 300 (13.6 ± 6.5), compared to time 0 (4.7 ± 1.4) in antibody treated animals (Figure 26).

Neutrophil Oxidant Production

PMNs obtained from septic animals at 300 min demonstrated a marked priming response for PMA-stimulated O_2^- production when compared to baseline PMNs, as noted by an increase in both rate of production and peak production of O_2^-. (Figure 27). In contrast, PMNs from control animals showed no priming over the course of

study. Pretreatment with anti-TNFα antibody failed to attenuate enhanced PMN short-lived oxidant generation. We found that PMNs obtained at 300 min from pretreated animals showed a similar degree of priming as that observed in septic animals.

Comparable findings were observed in PMN production of the long-lived oxidant, hypochlorous acid (HOCl). *Pseudomonas* sepsis resulted in significant priming of PMA-stimulated PMN HOCl production. This was not attenuated by pretreatment with anti-TNFα antibody (Figure 28) .

Extravascular Lung Water (EVLW)

EVLW was unchanged in saline controls throughout the study. Septic animals developed significant increases in EVLW and at 300 min EVLW reached 13.2 ± 1.5 ml/kg compared to 6.2 ± 0.9 at baseline. We observed no significant increase in EVLW in the antibody treated septic group over the course of the study (EVLW = 5.9 ± 0.7 ml/kg at 300 min) (Figure 29).

Bronchoalveolar Lavage Protein Analysis

The recovery of instilled BAL fluid at 0 min and 300 min was consistently high (> 70% return) and did not differ across groups. Baseline BAL protein content was similar in all three groups. In control animals, BAL protein content at 300 min did not differ from baseline (140 ± 18 vs 132 ± 21 μg/ml). In contrast, BAL protein content at 300 min in septic animals was more than 5-fold higher than baseline (770 ± 158 vs 137 ± 15 μg/ml, $p<0.05$). While anti-TNFα treated animals also showed an increase in BAL protein content at 300 minutes (313 ± 48 vs 141 ± 19 μg/ml), this was significantly less than that observed in septic animals (Figure 30).

Interleukin-6 Activity

TNFα which is known to be an important early mediator of gram-negative sepsis activates a complex cytokine cascade, of which the most important elements are likely interleukin-1 and interleukin-6. These cytokines are produced by, and act on, a wide range of tissues and are pleiotropic. Although interleukin-6 has been shown to be

elevated in many forms of tissue injury, the precise role of this hormone remains essentially unknown. Some workers regard it as an acute phase protein, as it shares many of the actions of this family of proteins. In this, however, it is no different from many other cytokines including TNFα and IL-1, both of which have been shown to cause pyrexia and act on the bone marrow to increase hemopoiesis. IL-6 is also a very potent inducer of acute phase protein synthesis in hepatocytes and is capable of surpressing albumin production by hepatocytes in favor of increased globulin production. It clearly has the capability to influence cellular metabolism in a major way.

In some studies looking at IL-6 levels in septic patients, extremely high levels are shown to correlate strongly with mortality and it has been suggested that this is a reflection of high TNFα levels in these patients. In the porcine model we have demonstrated an increase in IL-6 levels in septic animals which is not seen in saline control animals (Figure 31). In animals given monoclonal antibody to TNFα we demonstrated a marked attenuation of IL-6 levels, but these still remained slightly above levels observed in sham animals, suggesting that there are TNFα dependent and independent pathways for release of IL-6.

Combined Cyclooxygenase-Cytokine Blockade

Physiology of Porcine Sepsis

Systemic vascular resistance index (SVRI) rose sharply with the onset of bacterial infusion, peaking at 30 minutes (4647±508 dyne-sec/cm^5/m^2 30 min *vs* 2371±359 dyne-sec/cm^5/m^2, 0 min) in septic unprotected animals. SVRI subsequently returned to baseline at 60 minutes, where it remained until 180 minutes. It then showed a further moderate rise until the end of the study (3085±325 dyne-sec/cm^5/m^2, 300 min *vs* 2371±359 dyne-sec/cm^5/m^2, 0 min) (Figure 32). Animals pretreated with ibuprofen and anti-TNFα, showed no change in SVRI from baseline during the period of study and SVRI in this group was significantly less ($p < 0.05$) than that observed in septic animals during the *Ps* infusion (1960±97 dyne-sec/cm^5/m^2, at 30 min *vs*

4647±508 dyne-sec/cm^5/m^2).

Pulmonary vascular resistance index (PVRI) showed a response similar to that seen with SVRI (Figure 33). In septic animals, PVRI rose sharply with the onset of the bacterial infusion, peaking at 30 minutes (1563±249 dyne-sec/cm^5/m^2 at 30 min *vs* 256±39 dyne-sec/cm^5/m^2 at 0 min). It then briefly declined before rising again for the remainder of the study. Combined treatment animals maintained PVRI at or near baseline levels (345±45 dyne-sec/cm^5/m^2, 300 min *vs* 167±47 dyne-sec/cm^5/m^2, 0 min) for the duration of study and values were significantly less than those observed in unprotected animals for the entire study period (345±45 dyne-sec/cm^5/m^2, 300 min *vs* 1502±140 dyne-sec/cm^5/m^2, group I at 300 min).

Cardiac index fell precipitously in the septic group during the first 30 minutes following onset of sepsis (2.3±0.2 L/min/m^2 at 30 min *vs* 3.5±0.2 L/min/m^2 at 0 min), recovered slightly, before resuming a sustained decline to the end of the study (1.6±0.2 L/min/m^2 at 300 min *vs* 3.5±0.2 L/min/m^2 at 0 min). Combined treament animals showed a moderate increase in cardiac index in the initial 60 minutes of study (4.1±0.1 L/min/m^2 at 60 min *vs* 3.3±0.3 L/min/m^2 at 0 min). Cardiac Index subsequently declined for the remainder of the study period, but was not significantly different from control animals (2.5±0.1 L/min/m^2, *vs* 2.8±0.2 L/min/m^2, at 300 min) (Table 5).

Neutrophil Adhesion Receptor Expression and Kinetics

Neutrophil CD18 expression was studied at 60 min intervals. In septic animals, CD18 expression remained unchanged for the initial 60 min (Figure 34). Adhesion receptor expression subsequently rose sharply, peaking at 180 minutes (100±20 x 10^3 MESF *vs* 50±2.6 x 10^3 MESF, 0 min) before declining moderately prior to the end of the study. Neutrophil CD18 expression in the septic group was significantly greater than that observed in treatment animals from 120 minutes until the study conclusion (40±6.1 x 10^3 MESF, *vs* 90±10 x 10^3 at 300 min). There was no significant increase in neutrophil CD18 expression in combined treatment animals throughout the study

period.

Peripheral neutrophil count fell rapidly in septic animals following onset of sepsis, reaching a nadir by 120 minutes (3.6±0.3 x 10^3/µl at 120 min *vs* 24.5±1.3 x 10^3/µl, 0 min) and remaining depressed for the remainder of the study coincident with the rise in neutrophil CD18 expression (Figure 35). Neutrophil counts in treated animals fell significantly from baseline in the first 60 minutes of study (13.8±3.8 x 10^3/µl at 60 min *vs* 24.3±1.4 x 10^3/µl at 0 min). Combined treatment group neutrophil counts remained at this level and were significantly greater than neutrophil counts observed in unprotected septic animals for the remainder of the study (12.4±4.0 x 10^3/µl, *vs* 3.8±0.5 x 10^3/µl, at 300 min).

Coincident with the neutropenia observed, there was a marked increase in bronchoalveolar lavage (BAL) neutrophil content in septic animals at the study conclusion, which was almost 5 times greater than that observed at the study outset (29±3 at 300 min *vs* 6±1% at baseline). There was marked attenuation of neutrophil migration into the airspaces of the combined treatment group at the conclusion of the experiment (Figure 36).

Neutrophil Superoxide Generation

Neutrophils isolated from septic animals animals at the study conclusion showed evidence of significant priming for increased superoxide production over neutrophils isolated at baseline (22.5±3 nmol O_2^-/10^6 PMN/10 min at 300 min *vs* 13.2±3 nmol O_2^-/10^6 PMN/10 min at 0 min). Neutrophils isolated from combined treatment animals were also primed for significantly increased superoxide anion production, compared with baseline neutrophils (19.9±3 nmol O_2^-/10^6 PMN/10 min at 300 min, *vs* 7.3 nmol O_2^-/10^6 PMN/10 min at 0 min), (Figure 37). There was no difference in the degree of neutrophil priming at 300 min between septic and treated animals (19.9±3 nmol O_2^-/10^6 PMN/10 min, ComRx *vs* 22.5±3 nmol O_2^-/10^6 PMN/10 min, Septic)

Oxygenation and Alveolar-Capillary Membrane Integrity

Arterial pO_2 fell sharply in septic animals following onset of the pseudomonas infusion (Figure 38). This fall was sustained throughout the study period (67±5 Torr at 300 min *vs* 241±10 Torr at 0 min). In animals receiving combined pretreatment, p_aO_2 was maintained at baseline levels throughout the study period and did not differ from control at any time.

A significant increase was observed in bronchoalveolar lavage protein content (BAL-P) in septic unprotected animals at 300 minutes. The BAL-P content in this group was almost 5 times greater than baseline in the same group (904±123 μg/ml at 300 min *vs* 185±14 μg/ml at baseline). BAL-P content in the combined treatment group was not significantly different from baseline and was significantly less than BAL-P content in unprotected animals at 300 minutes (227±44 μg/ml, *vs* 904±123 μg/ml) (Figure 39).

60 Minute Post Treatment Anti-TNF Studies:

Plasma Tumor Necrosis Factor Activity. Plasma TNFα levels surged in septic animals, reaching a peak of 4.5 ± 0.5 U/ml at 120 minutes, and remained significantly elevated over baseline and control values at 300 min. Delayed anti-TNFα antibody treated animals showed an early significant increase in plasma TNFα activity, at 60 minutes (2.2 ± 0.6 U/ml) which returned to baseline and control levels at 120, 240 and 300 minutes.

Physiology of Porcine Sepsis and Effects of Delayed Anti-TNFa Antibody.

Septic animals exhibited significant cardiopulmonary derangements following onset of sepsis as described above. These derangements included early phase pulmonary arterial hypertension, rapidly developing systemic arterial hypotension associated with significant deterioration of cardiac output. In the latter phases of sepsis, unprotected animals failed to recover cardiac function and exhibited sustained pulmonary arterial hypertension, and systemic arterial hypotension, which was associated with an evolving metabolic acidosis and the development of a significant decline in PaO_2 and rise in $PaCO_2$, over the period of observation. Delayed anti-TNFα animals displayed

similar changes in pulmonary and systemic arterial pressures and decrements in cardiac index (Table 6), although there was evidence of late recovery in the systemic arterial pressure. Systemic pressure in unprotected septic animals fell significantly below control and baseline at 240 and 300 minutes, whereas systemic pressure in delayed anti-TNFα animals recovered somewhat and was not significantly different from control or baseline at the same time points. In addition, despite an early and significant fall in arterial oxygen tension, which persisted throughout the study, delayed anti-TNFα animals retained significantly improved and recovering arterial oxygen tensions at 180 240 and 300 min compared with unprotected septic animals ($p < 0.05$ respectively, Figure 40).

Similarly, arterial pH in delayed anti-TNFα animals fell significantly at 120 minutes, then recovered to control levels by 240 and 300 min (Figure 40).

Bronchoalveolar Lavage Protein Analysis. BAL protein content at 300 min in septic animals was more than 7-fold higher than baseline (149 ± 28 vs 1044 ± 270 μg/ml, $p < 0.05$), while delayed anti-TNFα animals showed no significant increase in BAL protein content at 300 minutes (129 ± 19 vs 217 ± 83 μg/ml), (Figure 41).

Peripheral White Cell Counts. Circulating white blood cell (WBC) counts fell by more than 80% in septic animals and remained depressed throughout the study period. Treatment with anti-TNFα antibody dramatically altered the WBC profiles producing a biphasic response (Figure 42).

Neutrophil CD11/CD18 Expression. PMNs obtained from septic animals exhibited significant upregulation of CD11/18 expression compared to baseline and control values, from 120 to 300 minutes (Figure 43). In contrast, delayed anti-TNFα animals showed significant upregulation of CD11/18 expression compared to base line at 120 and 180 min only ($59x10^3 \pm 3x10^3$ baseline, $103x10^3 \pm 11x10^3$ 120 min, $96x10^3 \pm 7x10^3$ 180 min, $p < 0.05$ respectively).

Lung Myeloperoxidase Activity. In delayed anti-TNFα animals, treatment significantly

reduced MPO activity and hence lung PMN burden when compared to unprotected animals (34.6 ± 4.7 vs 50.5 ± 4 U/g, $p < 0.05$), but retained significantly higher MPO activity than controls (34.6 ± 4.7 vs 11 ± 5 U/g, $p < 0.05$) (Figure 44).

Neutrophil Transendothelial Migration. There was a significant increase in PMNs recovered from BAL at 300 min (10 ± 3) compared to 0 min (1 ± 0.5) in delayed anti-TNFα animals, but this increase was significantly less than in the septic group at 300 min ($p < 0.05$) (Figure 45).

Neutrophil Oxidant Production. PMNs obtained from septic animals at 300 min demonstrated a marked priming response for PMA-stimulated O_2^- production when compared to baseline PMNs, as noted by an increase in total production of O_2^- over the 10 minute assay period (4.0 ± 0.7 vs 15.3 ± 3. $p<0.05$). Delayed treatment with anti-TNFα antibody failed to attenuate enhanced PMN short-lived oxidant generation (Figure 46).

90 Minute Post Treatment Anti-TNFα Studies

None of the parameters examined in this study were significantly different from those in the unprotected septic animals and it was therefore decided to terminate the study as ineffective. Thus a therapeutic window was established for usage of this agent, showing efficacy only, up to 60 minutes after onset of sepsis.

Soluble Complement Receptor

The cohort of animals treated with sCr1 were compared with results obtained in control and septic animals, previously studied. There were no significant differences between the septic animals and those treated with sCR I with respect to any parameter measured, including systemic arterial pressure, pulmonary arterial pressure, cardiac index, arterial pO_2, arterial pH and extra-vascular lung water.

Nitric Oxide Changes in Anti-TNFαTreated Animals

Hemodynamic Variables

Pretreatment with Anti-TNF-α MoAb significantly attenuated the decrease in systemic arterial pressure and the fall in cardiac index observed in septic animals. The nadir of systemic arterial pressures and SVR in septic animals coincided with peak levels nitrite. There were no significant differences in left ventricular preload between groups throughout the study. Septic animals demonstrated a decrease in cardiac index especially in the mid and late period of the experiment. Animals that were pretreated with the anti-TNF-α MoAb did not have significant decreases in Cardiac Index in the mid and latter periods of the protocol.

EDRF Activity

Septic (untreated) animals displayed a rise in blood nitrite (EDRF equivalent) which peaked at 2 hours (0.454 ± 0.074 μM at 2 hrs vs 0.189 ± .094 μM at zero hr) into the study (Figure 47). Antibody treated animals showed significantly higher levels of nitrite (3.658 ± 1.75 μM at 1 hr vs 0.193 ± 0.10 μM at baseline) with blood levels peaking at 1 hour earlier than that observed in septic untreated animals. Antibody treated septic animals exhibited sustained, significant elevation in blood nitrite throughout the study when compared to both septic and control groups (0.685 ± 0.356 μM at 5 hrs in anti-TNF group vs 0.218 ± 0.094 μM at 5 hrs in septic group vs 0.027 ± 0.017 μM at 5 hrs in control group). Nitrite levels in the *Pseudomonas* infusate and in the Anti-TNF-α MoAb were negligible and did not contribute to elevated circulating

nitrite.

Endothelin Activity

Septic animals exhibited a slow but significant rise in plasma endothelin levels which peaked at 3 hours (65.37 ± 28.32 pg/ml at 3 hrs vs 10.45 ± 1.82 pg/ml at zero hr) (Figure 48). Anti-TNFα pretreatment significantly attenuated the rise in plasma endothelin and significantly blunted the peak of endothelin at 3 hours (24.06 ± 6.84 pg/ml at 3 hrs in anti-TNFα group vs 64.37 ± 28.32 pg/ml at 3 hrs in septic group). Control animals exhibited stable endothelin levels (13.57 ± 4.05 pg/ml at zero vs 10.88 ± 2.72 pg/ml at 5 hrs) which did not change significantly during the study.

Conclusions

General

The data reviewed above can be roughly subclassified into two categories, those pertaining to hemodynamic derangments of gram negative sepsis and those pertaining to the evolution of acute lung injury. It is undoubtably true that these two areas of focus are intimately associated, but for the purposes of a coordinate and comprehensible discussion they will be dealt with seperately. In addition, it was the intention of the authors to conduct the studies in two ways. Firstly the pretreatment studies attempted to elucidate some of the mechanisms behind the derangments noted in the model. Secondly, the post treatment studies were aimed at a more clinically relevant scenario of therapeutic intervention following the onset of bacterial sepsis.

Hemodynamic Variables

It is apparent from the pretreatment ibuprofen data that, early changes in hemodynamic variables are mediated by products of cyclooxygenase metabolism. The acute spike in pulmonary artery pressure occuring at 30 minutes post sepsis, with the concomitant fall in cardiac output and arterial oxygen tension are associated with an acute rise in plasma thromboxane levels. Further, the pre-administration of ibuprofen inhibited this thromboxane spike and attenuated early hemodynamic changes.

In contrast, the pretreament anti-TNFα data suggested that the later changes in hemodynamic variables were mediated by cytokines. The persistent elevation in pulmonary arterial pressure, the decline in cardiac output, systemic arterial pressure, arterial pH and increase in EVLW, began with the maximal elevation of TNFα at approximately 120 minutes and continued to the completion of the study. All these late changes were attenuated to some degree by the pre-administration of the monoclonal antibody to TNFα.

To confirm these apparent findings, that the evolution of the septic response in this model relied on the sequential release of these key humoral mediators, the

combined anti-TNFα and ibuprofen study was conducted. The results in this study confirmed the phasic nature of sepsis in this model, demonstrating inhibition of both the early and late hemodynamic derangments described above. Indeed it is to be noted that, in the combined treatment regimen, none of the hemodynamic variables studied were noted to be significantly worse than those of the non septic control animals.

The global protection afforded by these two agents belies the complexity of the septic response. It is known that a wide variety of other hunmoral mediators are involved in the evolution of sepsis and their importance has been stated in other publications. To this end we attempted to look at complement and its role in gram negative sepsis, by utilizing an agent that inhibits both the classical and alternate pathways of complement activation. sCr1 administered in this model showed no significant benefit in terms of hemodynamic protection, despite laboratory tests conducted by the makers of sCr1 that indicated that the agent had, *in vivo*, blocked the activation of porcine complement. These results serve to strengthen the growing evidence that despite multiple other humoral mediators of sepsis, both the eicosanoid and cytokine systems appear to be pivotal and essential to the normal septic response.

Vascular endothelium was, at one time, considered to play a passive role and yet be the ultimate victim in sepsis. Recent evidence has, however, suggested that the endothelium does in fact play a critical role in the normal physiological and pathological control of vascular tone and thus systemic and pulmonary blood pressure. Results from the nitric oxide study, demonstrated elevated EDRF and endothelin levels in septic animals. Surprisingly, the administration of anti-TNFα, while improving systemic blood pressure actually dramatically increased levels of both EDRF and endothelin above those seen in unprotected sepsis. These results suggested that vascular tone is not governed by absolute levels of EDRF and endothelin, but rather they are involved in a complex counter regulatory loop that regulates tone and thus pressure in sepsis.

White Cell Kinetics

The current studies have also focused on the activation, sequestration and migration of PMNs. It is the opinion of the authors that PMNs are the key cellular mediators of the host tissue injury that is manifest in the ARDS like lung injury seen in the experimental model. Several lines of evidence have supported a microenvironemnt theory of PMN mediated tissue injury in sepsis. This theory suggests that adherence of activated PMNs to endothelium creates an intercellular microenvironment into which PMNs secrete toxic products such as reactive oxygen metabolites and proteinases and that this microenvironment is protected from the action of circulating oxidant scavengers and proteinase inhibitors permitting unchecked endothelial injury. From the point of view of mechanisms, it seems that the microenvironment theory requires two fundamental neutrophil actions. 1) upregulation of integrin (CD11/18) adhesion receptors and thus, removal from the circulation and sequestration within lung and other organs, adherent to the endothelium. 2) priming of PMN metabolic functions that allow generation of oxygen radicals and release of proteases that mediate the tissue injury.

Once again the data from the above studies all provide corroborative evidence to support this theory of PMN mediated tissue injury and in particular acute lung injury. Ibuprofen administration was associated with a significant improvement in lung injury evidenced by reduced BAL protein concentration reduced EVLW and improved arterial oxygen tension. In addition, ibuprofen prevented priming of PMNs for oxygen radical production and inhibited lysosomal enzyme release, one of the limbs of the microenvironment theory. Both anti-CD18 and anti-TNFα also attenuated acute lung injury associated with this model and both inhibited CD18 function, preventing loss of PMN from the circulation reducing PMN lung sequestration and PMN transendothelial migration, the other limb of the microenvironment theory.

The other cells of interest to the laboratory have been the pulmonary

intravascular macrophage. PIMs are atypical cells that sit, adherent to the circulatory side of the alveolar capillary membrane, possessing all the phagocytic and metabolic functions of other tissue macrophages and yet are uniquely positioned to sample the local circulatory conditions and respond accordingly. An original finding was the TGFß response of the PIM to LPS and the suggestion that this cell was not only involved in the inflammatory response but also in resolution and repair following the septic insult. Unfortunately although this cell had many fascinating and potentially therapeutically maleable properties, attempts at isolating them in the human proved essentially impossible and therfore further research was suspened.

Delayed Treatment Studies

The exceptional cardiopulmonary protection afforded by most of the agents examined in this laboratory was encouraging and yet lacked true clinical relevance. Hence, administration of both ibuprofen and anti-TNFα was delayed until sepsis was established in an attempt to mimic a clinical scenario. In both cases, a period of delay prior to administration reduced the efficacy of the drug in terms of cardiopulmonary protection, but nonetheless both agents provided some significant degree of protection over unprotected sepsis. These data suggested therfore that these agents did have an effective therapeutic window during which time they may retain some efficacy. In an attempt to define the temporal limits of this window the period of delay was extended in the anti-TNFα studies and unfortunatley at 90 minutes following the onset of sepsis the agent lost all therapeutic benefit. Recent clinical trials of both these agents have had similarly dissapointing results, indeed, neither agent significantly altered mortality compared to placebo when given to patients with sepsis.

Future Directions

The specific inhibition of mediators whose apparent systemic release initiates a cascade of events culminating in the devastating syndrome of sepsis, is an attractive idea. It is however, not without potential detrimental side effects. The existence of such

an organized and conserved cascade begs the question, "what are the beneficial effects of this system ?" The cellular and chemical response to sepsis is an integral part of the host immune response. Inhibiting or altering this response may attenuate the resultant host injury but may also expose the host to overwhelming secondary infection. For example, activation of neutrophils is a necessary step in enhancing their primary role of antimicrobial defence. It is apparent from the studies in this report that inhibition of this activation using anti-CD18, anti-TNFα antibody or ibuprofen prevents some of the detrimental side effects of activated neutrophils. What is not apparent however is the longer term effects of this inhibition in terms of risk to the host of secondary infection. This risk is illustrated by patients in whom neutrophils are deficient in the common CD18 subunit of the surface adhesion receptor, a defect known as leukocyte adhesion deficiency (Arnaout, 1990). These patients suffer recurrent bacterial infection and an increased mortality. Animal studies have also demonstrated that use of the monoclonal antibody to the CD18 receptor increase the formation of metastatic abscesses on exposure to high dose bacterial innoculation (Sharar et al.1991). As a consequence of this potential side effect there has been increasing interest in attempting to modify the end-organ response to neutrophil mediated tissue injury without affecting neutrophil or immune function. Drugs which stabilize the endothelial monolayer by modifying the cellular cytoskeleton, have been shown to attenuate permeability injury in lung and skeletal muscle following ischemia/reperfusion (Goldman et al.1991; Korthuis et al.1991). The effect of these drugs in sepsis are currently under investigation in this laboratory and the results are encouraging. It may also be possible to increase the endothelial cell's ability to withstand oxidant injury for instance by upregulating intracellular glutathione metabolism using gene therapy, another avenue being persued currently in the laboratory.

An alternative approach to the temporal constraints of the therapeutic window, apparent in the experimental and clinical studies, would be to develop a method of

early risk prediction. If it were possible predict those patients in whom ARDS and multiorgan dysfunction were going to develop, these patients could be targetted for aggressive intervention. This laboratory has some extremely exciting new data from preliminary studies looking at levels of shed endothelial adhesion receptors in the circulation of septic patients in the intensive care unit. These data indicate that the level of circulating intercellular adhesion molecule-1 (cICAM) correlates well with the development of both multi-organ dysfunction and mortality. These studies are currently being extended to other patient populations and their application modified in an attempt to establish a predictive value for cICAM.

In addition to these new approaches to the managment of ARDS and sepsis, the laboratory continues to examine other novel agents in both a pretreatment and post treatment fashion, to probe deeper the mechanisms of the septic response and to evaluate the potential benefit of these agents in clinical practice.

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Table 3. Ibuprofen, Pretreatment and Delayed

Hemodynamic Data

		0 hr	0.5 hr	1 hr	2 hr	3 hr	4 hr	5 hr
CI (L/min /m^2)	Control	3.3±0.2	3.5±0.2	3.3±0.2	3.2±0.2	3.1±0.2	2.9±0.1	2.9±0.1
	Septic	3.1±0.2	2.1±0.2*#	2.5±0.3#	2.5±0.2#	2.1±0.1*#	1.7±0.1*#	1.5±0.1*#
	Ibu	3.1±0.4	3.1±0.3	3.3±0.3	2.9±0.3	2.4±0.2*	2.2±0.3*	1.8±0.2*
	Ibu-D	3.2±0.2	2.7±0.3	3.0±0.3	2.7±0.2	2.4±0.3*	1.9±0.1*	1.7±0.7*
PAP (mmHg)	Control	12.9±1.0	13.9±1.1	15.4±1.0	15.4±0.9	16.5±1.4#	16.1±1.2	15.9±0.9
	Septic	14.9±1.2	46.3±1.6*#	36.6±1.5*#	29.0±1.4*#	32.0±1.7*#	32.6±2.2*#	30.7±2.9*#
	Ibu	14.6±0.8	19.3±1.8#	27±3.3*#	24.8±1.4*#	23.5±1.9*#	25.8±2.4*	28.8±2.2*
	Ibu-D	13.2±1.0	47.3±2.0*	16.5±1.0#	19.2±2.0#	20.5±2.0*#	24.6±2.0*	26.7±3.0*
SAP (mmHg)	Control	94.6±2.1	99.4±3.5	105±3.5	110±3.1#	110±3.0#	105±6.4	110±4.8#
	Septic	98.7±5.4	133±5.5*#	98.0±8.1	76.6±6.8*#	78.8±5.9*#	78.1±4.6*#	78.3±7.1*#
	Ibu	104±3.0	116±5.0*	125±5.0#	111±7.0#	104±7.0#	100±8.0	114±10#
	Ibu-D	104±9.0	131±3.0*	120±6.0#	108±8.0#	110±8.0#	113±10#	116±9.0#
PaO_2 (mmHg)	Control	250±8.0	247±10.0	263±8.0	251±7.0	246±8.0	255±9.0	252±9.0
	Septic	242±8.0	150±8.0*#	107±8.0*#	108±9.0*#	84±6.0*#	72±6.0*#	68±5.0*#
	Ibu	242±12	250±14#	241±27#	253±14#	197±38#	171±33*#	134±38*#
	Ibu-D	261±7.0	209±12#	220±9.0*	250±12#	248±12#	218±11*#	186±17*#

** = P<0.05 vs Control* *# = p<0.05 vs Baseline*

Table 4. Pretreatment Anti-TNFα

Hemodynamic Data

		0 hr	0.5 hr	1 hr	2 hr	3 hr	4 hr	5 hr
CI	Control	3.3±0.2	3.5±0.2	3.3±0.2	3.2±0.2	3.1±0.2	2.9±0.1	2.9±0.1
(L/min. /m^2)	Septic	3.1±0.2	2.1±0.2*#	2.5±0.3#	2.5±0.2#	2.1±0.1*#!	1.7±0.1*#!	1.5±0.1*#!
	Anti-TNF	3.9±0.4	2.4±0.2*#	2.6±0.3#	3.1±0.3	2.9±0.3	2.3±0.2*#	2.6±0.3#
PAP	Control	12.9±1.0	13.9±1.1	15.4±1.0	15.4±0.9	16.5±1.4#	16.1±1.2	15.9±0.9
(mmHg)	Septic	14.9±1.2	46.3±1.6*#	36.6±1.5*#	29.0±1.4*#	32.0±1.7*#!	32.6±2.2*#!	30.7±2.9*#!
	Anti-TNF	11.3±0.8	41.7±2.4*#	35.7±2.8*#	24.7±1.6*#	23.0±1.1*#	22.8±2.2#	23.5±1.6#
SAP	Control	94.6±2.1	99.4±3.5	105±3.5	110±3.1#	110±3.0#	105±6.4	110±4.8#
(mmHg)	Septic	98.7±5.4	133±5.5*#!	98.0±8.1	76.6±6.8*#	78.8±5.9*#	78.1±4.6*#	78.3±7.1*#
	Anti-TNF	91.2±2.2	103.7±6.4	102.5±5.3	88.3±4.9	90.0±3.7	90.0±3.4	99.2±6.5

** = P<0.05 vs Control, # = p<0.05 vs Baseline, ! p<0.05 vs Anti-TNFα*

Table 5. Combined Ibuprofen and AntiTNFα

Hemodynamic Data

		0 hr	0.5 hr	1 hr	2 hr	3 hr	4 hr	5 hr
CI	Control	3.6±0.2	3.5±0.3	3.2±0.2	3.0±0.2	3.0±0.2	2.9±0.2	2.9±0.2
(L/min /m^2)	Septic	3.5±0.2	2.2±0.2$^{*\#\phi}$	2.8±0.3	2.8±0.2	2.5±0.1$^{\#}$	2.0±0.1$^{*\#}$	1.6±0.1$^{*\#}$
	Comb	3.3±0.4	4.0±0.2*	4.0±0.3	2.6±0.3	2.9±0.3	2.4±0.2$^{\#}$	2.5±0.3$^{\#}$
PAP	Control	13.8±1.0	14.0±1.0	15.2±1.0	15.6±0.9	15.6±1.4	15.1±1.2	15.8±1.3
(mmHg)	Septic	14.9±1.2	47.0±1.6$^{*\#\phi}$	36.2±2.5$^{*\#}$	30.0±1.0$^{*\#\phi}$	33.5±1.4$^{*\#\phi}$	34.4±1.7$^{*\#\phi}$	33.1±1.7$^{*\#\phi}$
	Comb	14.2±1.5	17.2±1.8	25.0±3.9$^{*\#}$	27.0±1.8	22.0±1.7	23.4±1.7$^{*\#}$	27.5±2.3$^{*\#}$
SAP	Control	98.3±4.1	98.1±3.4	107±4.0	115±4.8$^{\#}$	122±4.7$^{\#}$	113±6.2$^{\#}$	120±5.3$^{\#}$
(mmHg)	Septic	102±5.9	128±4.4$^{*\#\phi}$	96.1±8.4	76.6±5.6$^{*\#}$	78.0±4.5$^{*\#}$	77.1±2.6$^{*\#}$	77.5±6.1$^{*\#}$
	Comb	98.0±4.0	98.0±3.7	99.2±6.7	96.6±4.0	84.6±5.3*	81.0±8.0	95.4±4.0

** = P<0.05 vs Control, # = p<0.05 vs Baseline, ϕ = p<0.05 vs Combined*

Table 6. Post Treatment Anti-TNFα.

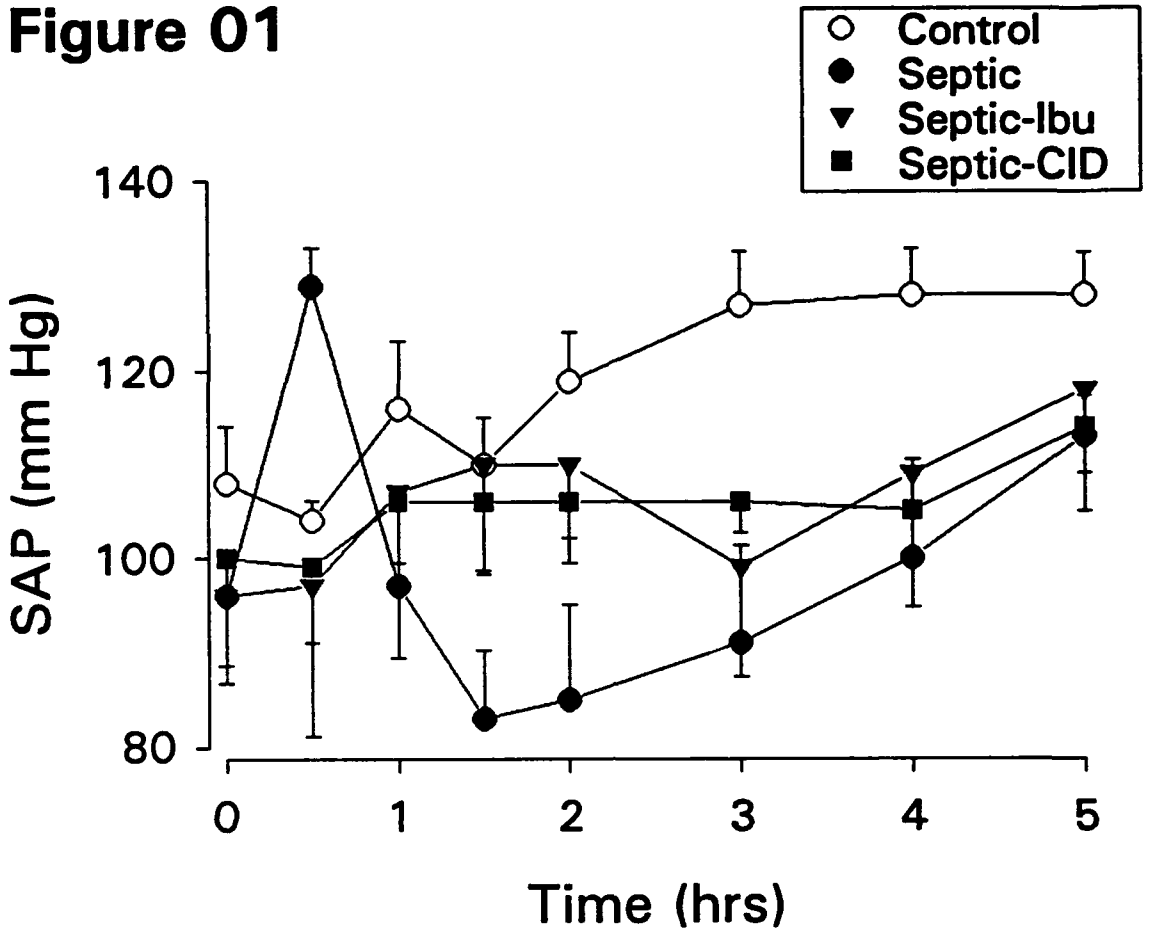
Hemodynamic Data

		0 hr	0.5 hr	1 hr	2 hr	3 hr	4 hr	5 hr
CI	Control	3.5±0.2	3.4±0.3	3.1±0.2	3.0±0.1	3.0±0.1	2.9±0.1	2.8±0.1
(L/min /m^2)	Septic	3.4±0.2	2.2±0.2*	2.8±0.2	2.8±0.3	2.5±0.2	2.0±0.1*	1.6±0.2*
	TNF	2.9±0.2	2.0±0.3*	2.6±0.2	2.6±0.2	2.2±0.2	2.0±0.3*	2.0±0.3*
PAP	Control	13.8±0.9	14.0±1.0	15.2±0.9	15.6±0.9	15.6±1.1	15.0±1.3	15.9±1.2
(mmHg)	Septic	14.0±1.2	48.0±1.7*	37.0±2.0*	31.0±1.5*	36.0±1.6*	35.0±1.6*	34.0±1.8*
	TNF	16.0±1.0	48.0±2.3*	40.0±2.4*	32.0±2.4*	36.0±2.5*	34.0±2.1*	34.0±1.2*
SAP	Control	97.3±4.0	97.3±3.2	106±3.8	115±4.0	121±4.5	113±4.7	117±5.7
(mmHg)	Septic	101±5.4	126±4.6*	95.0±7.6	76.6±6.1*	78.5±5.3*	80.3±4.7*	76.4±6.6*
	TNF	95±5.0	109±6.0	84.0±5.9	74.3±8.7*	89.0±11*	98.0±9.6	97.2±13

** = P<0.05 vs Control # = p<0.05 vs Baseline*

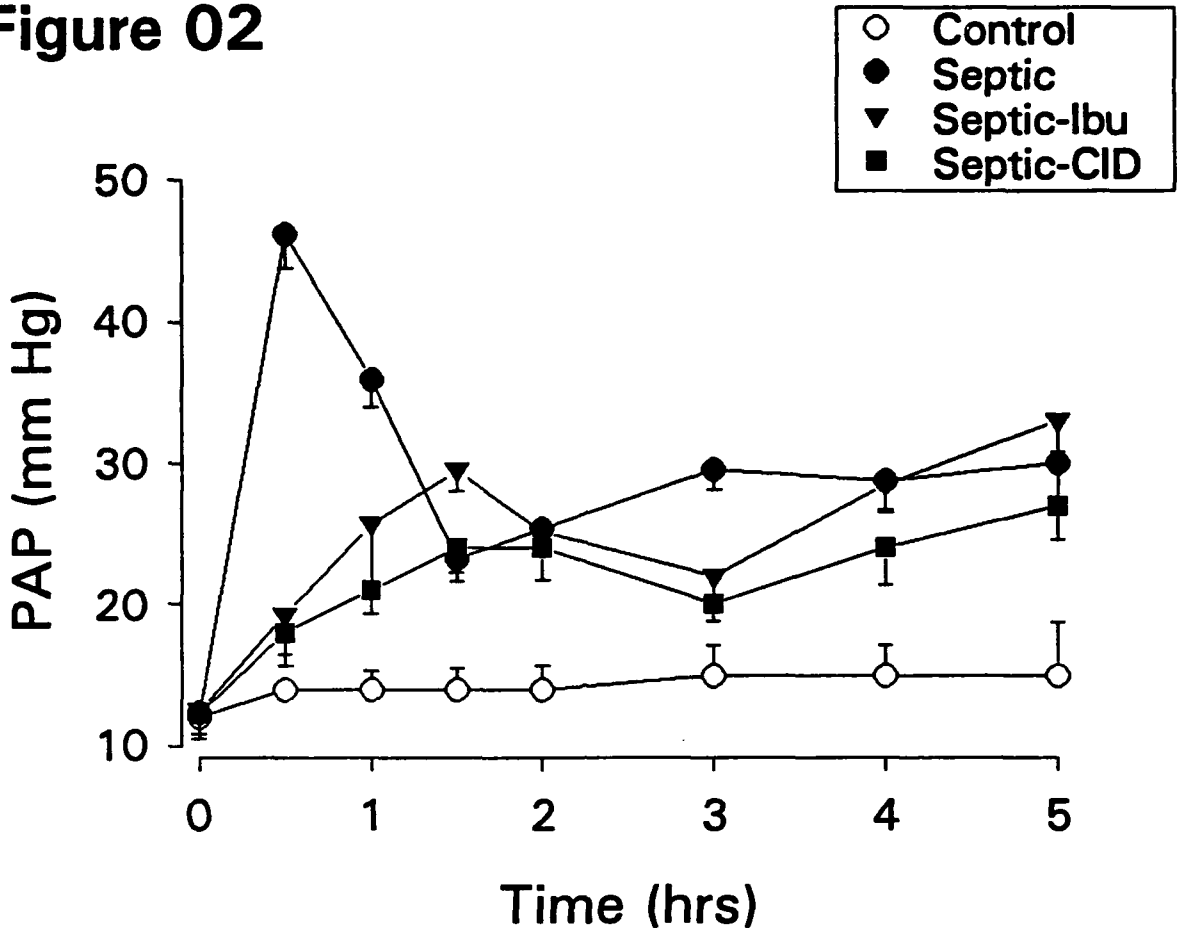
! = p<0.05 vs Anti-TNF

Figure 01



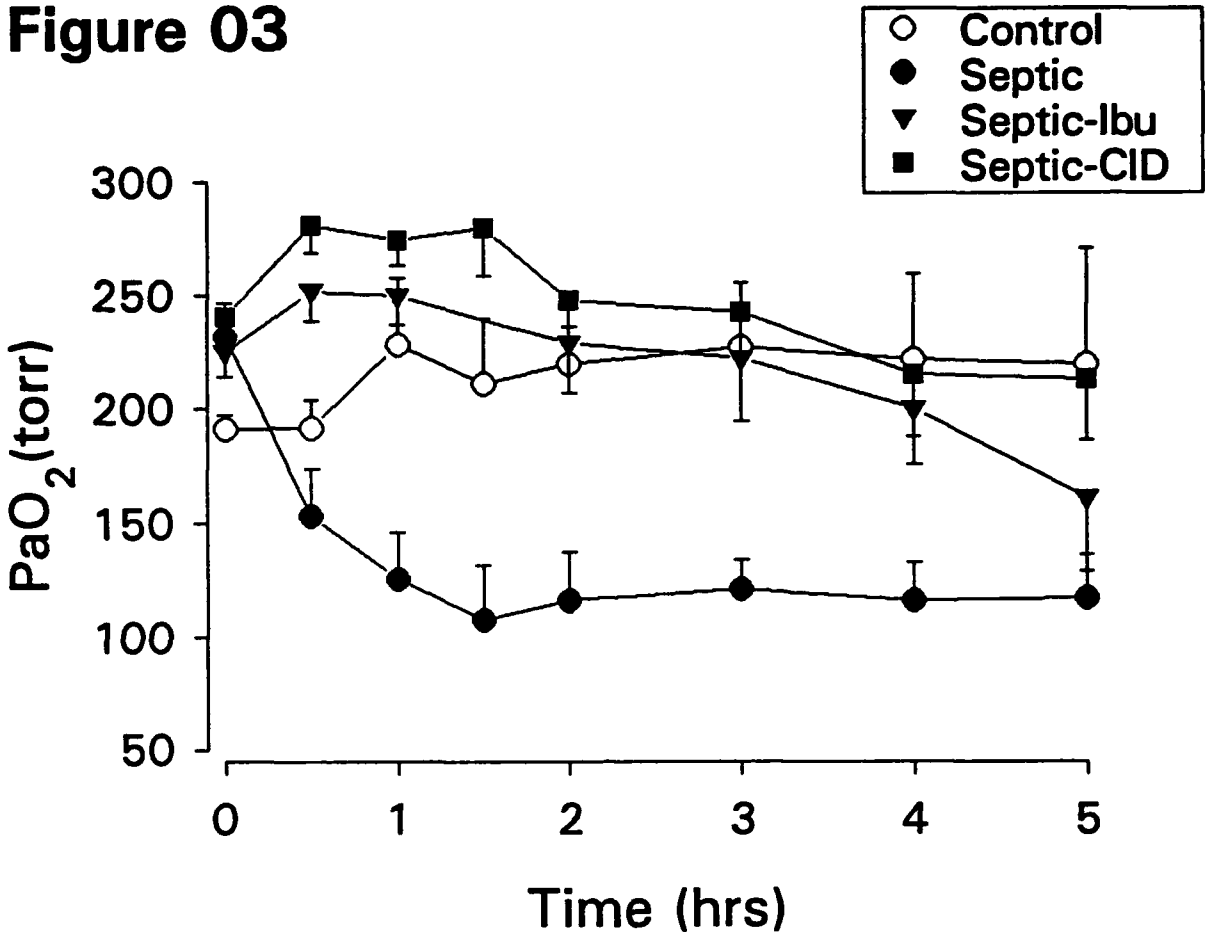
Systemic Arterial Pressure. Ibuprofen and combined agents attenuated arterial hypotension associated with porcine sepsis.

Figure 02



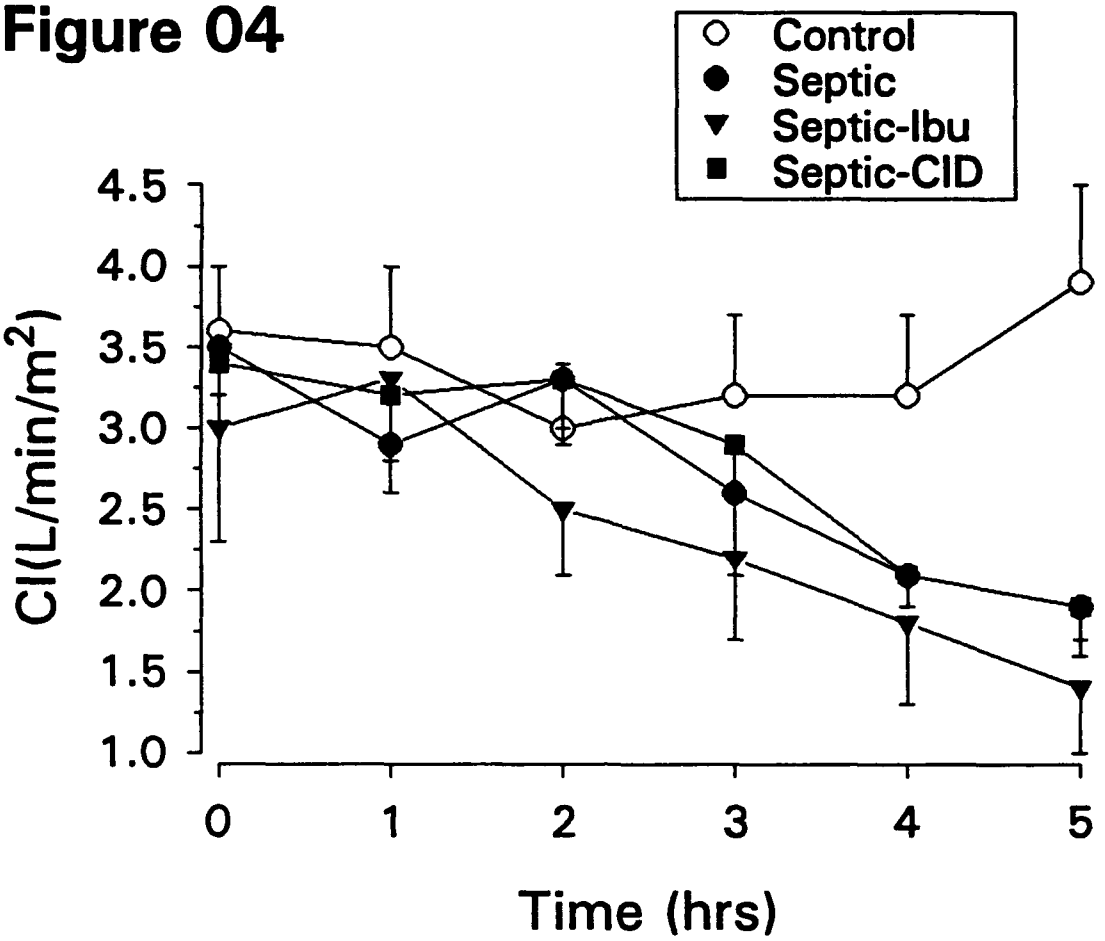
Pulmonary Arterial Pressure. Ibuprofen and combined agents (cimetedine, ibuprofen, diphenhydramine) attenuate early phase pulmonary arterial hypertension, but fail to affect late phase pulmonary hypertension.

Figure 03



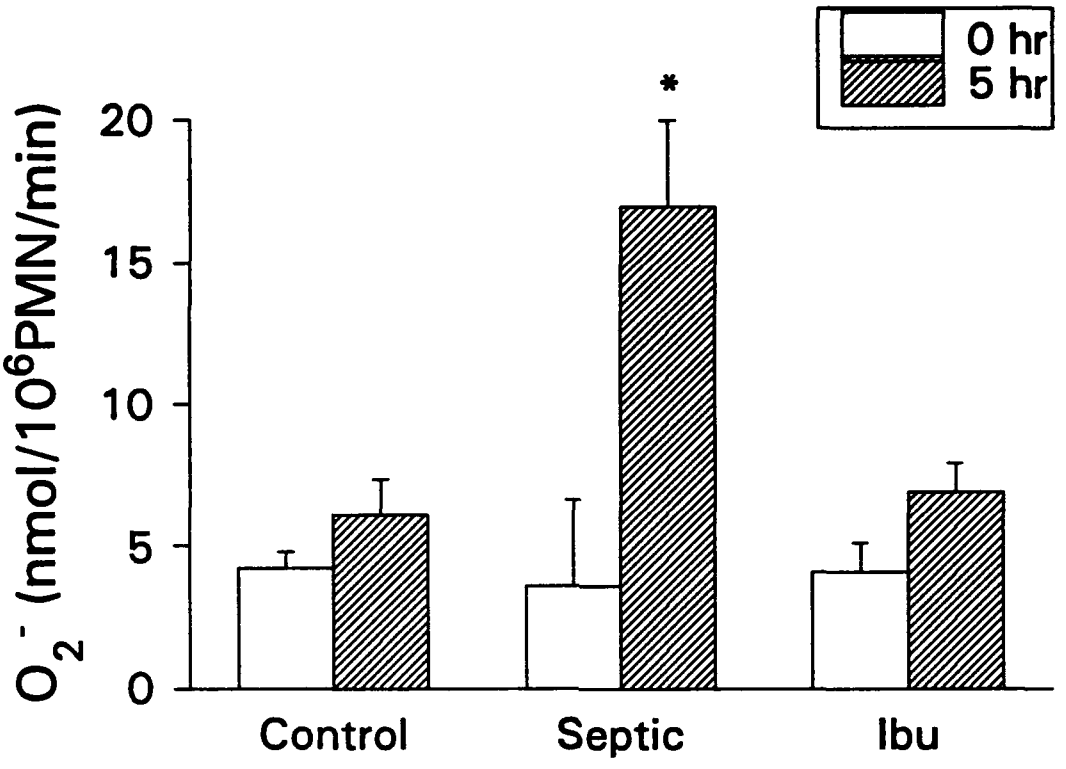
Arterial Oxygen Tension. When compared to ibuprofen alone, combined agents preserved arterial oxygen tension throughout the period of study. By completion of study, oxygenation in animals treated with IBU alone was not different from septic untreated animals.

Figure 04



Cardiac Index. Neither IBU or combined agents prevented left ventricular function from deteriorating over the course of 5 hours of sepsis

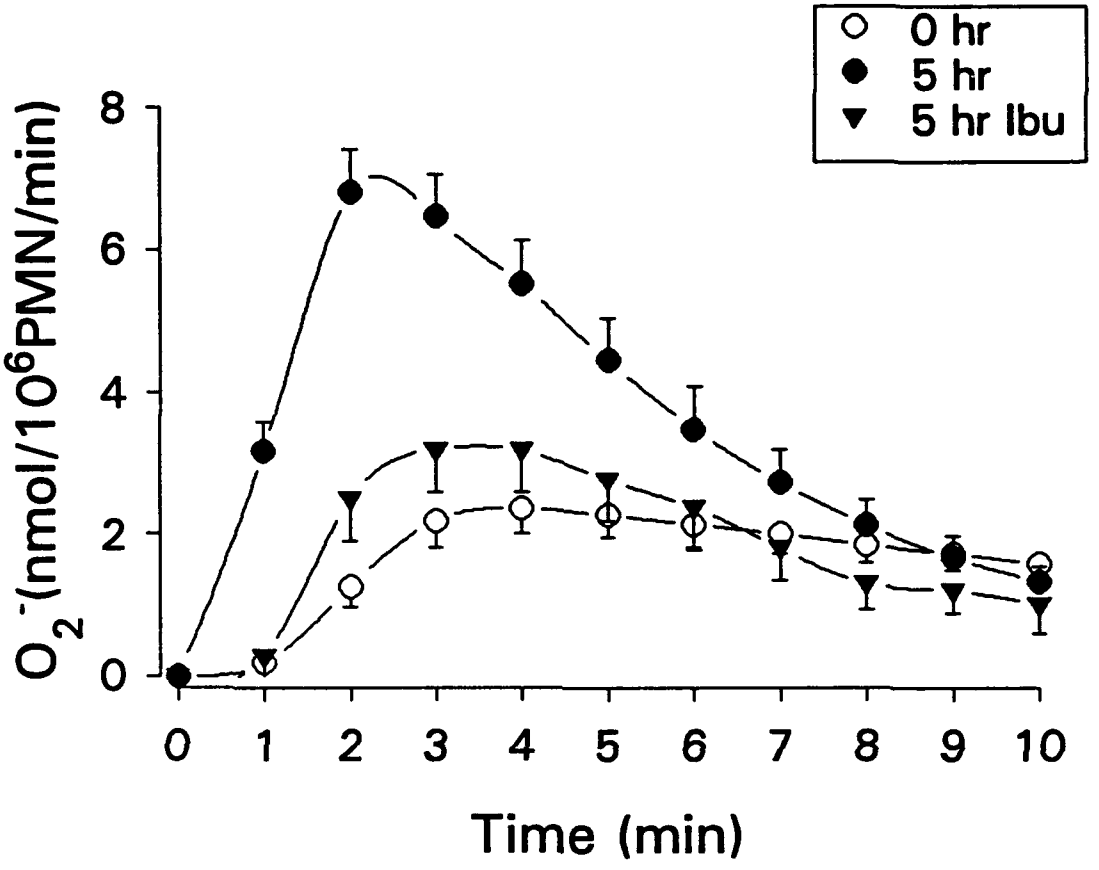
Figure 05



*$p<0.05$ vs 0 hour

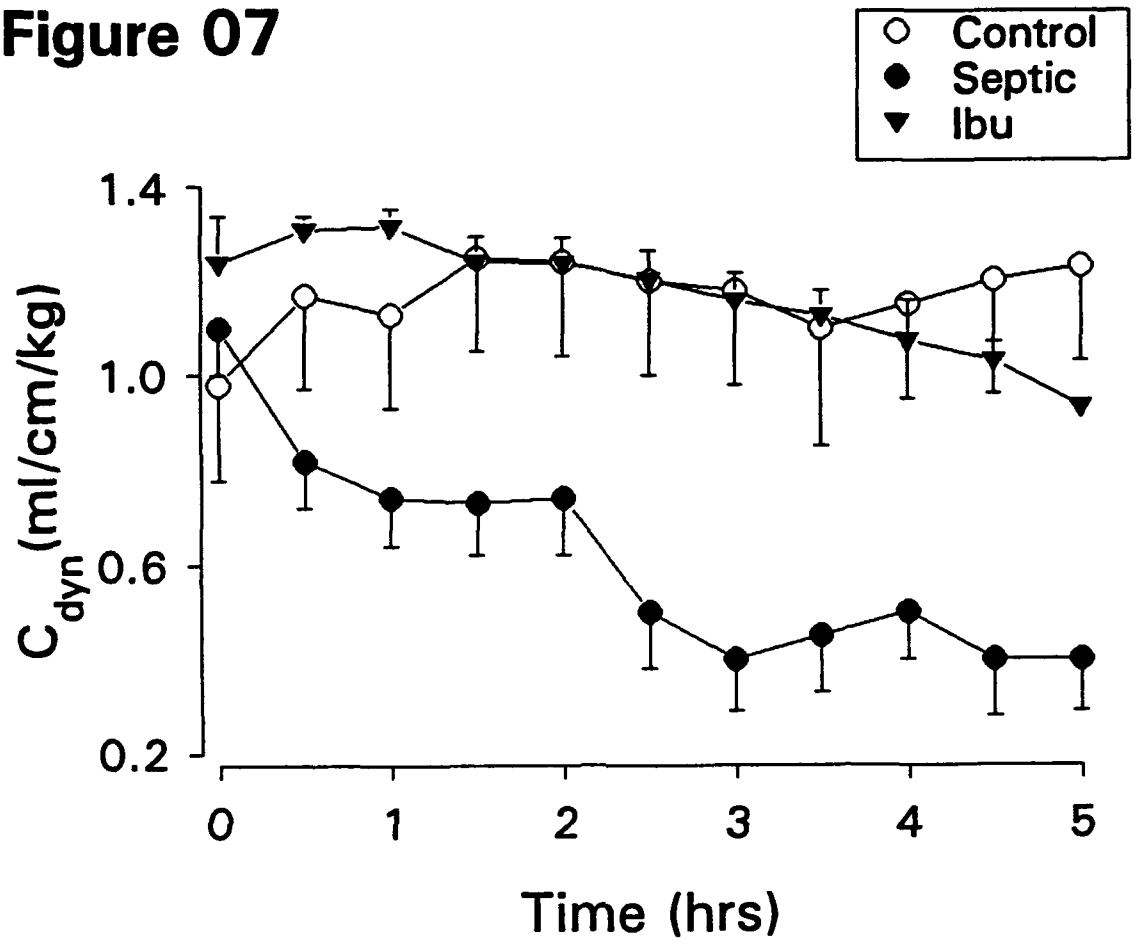
Neutrophil Superoxide Anion. Ibuprofen attenuated neutrophil oxidant priming associated with sepsis

Figure 06



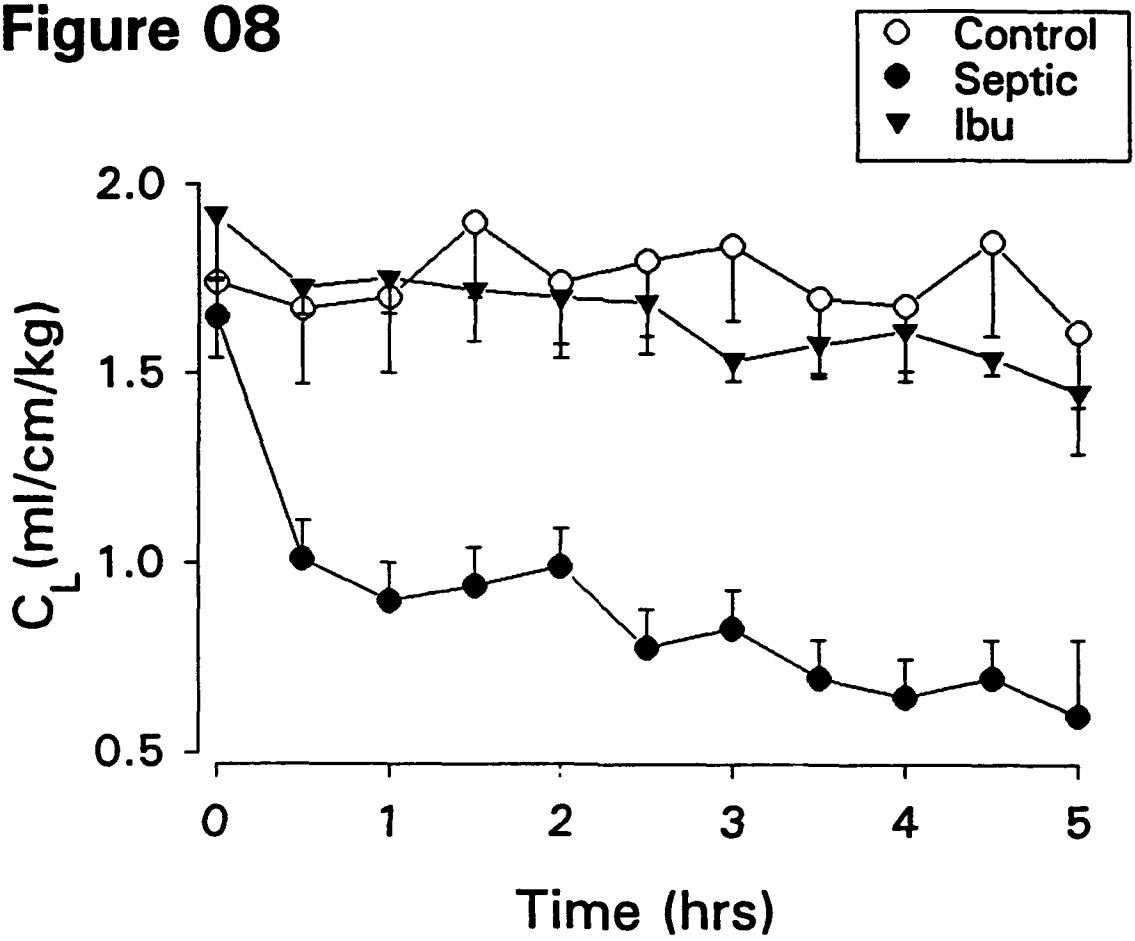
Neutrophil Superoxide Kinetics. Kinetics analysis of PMA stimulated superoxide anion generation showed that IBU significantly attenuated the oxidant priming associated with sepsis.

Figure 07



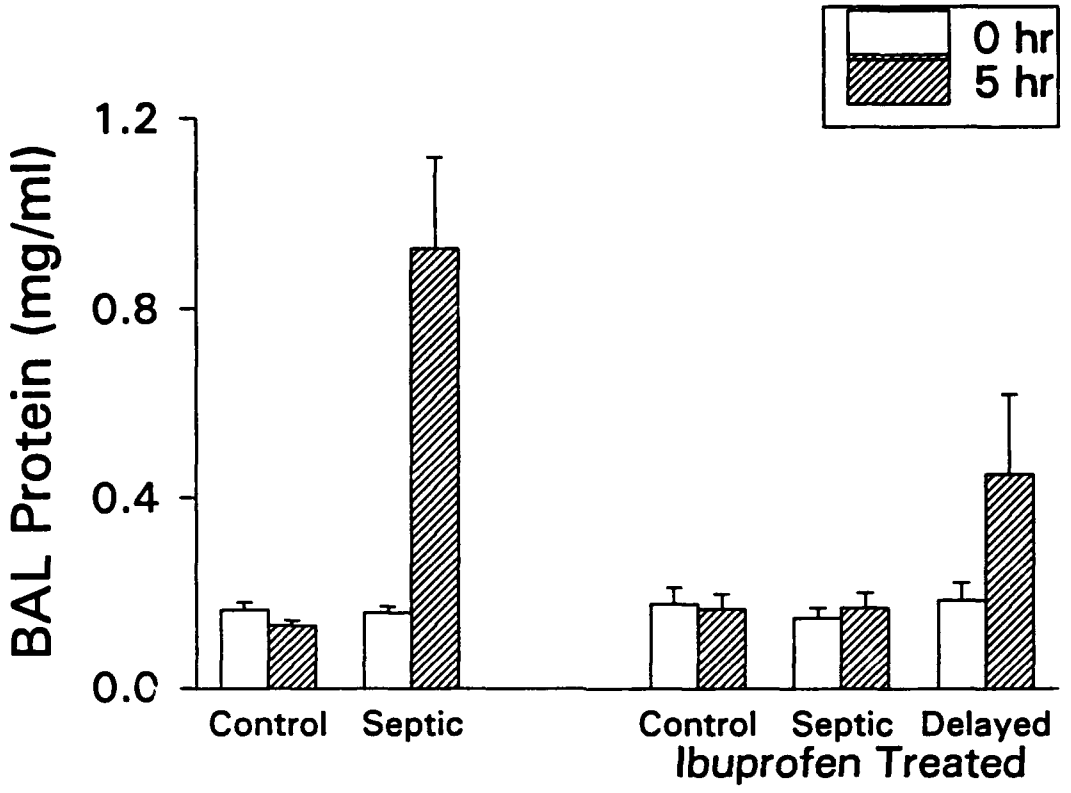
Dynamic lung Compliance. Septic animals exhibited rapid onset of diminished dynamic compliance. IBU administration preserved dynamic compliance at baseline and during the course of sepsis.

Figure 08



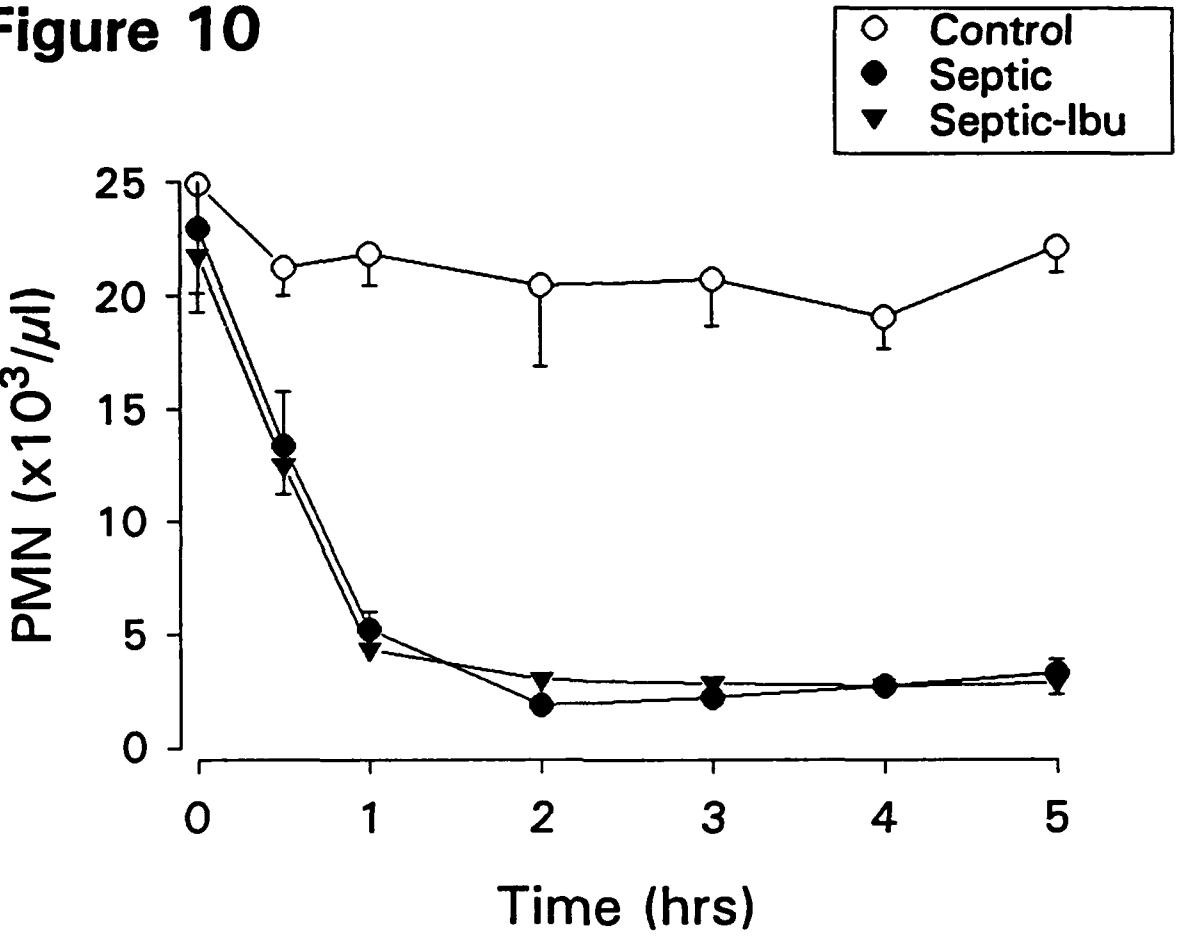
Static Lung Compliance. Sepsis was associated with falling static lung compliance. IBU administration maintained lung compliance at baseline values.

Figure 09



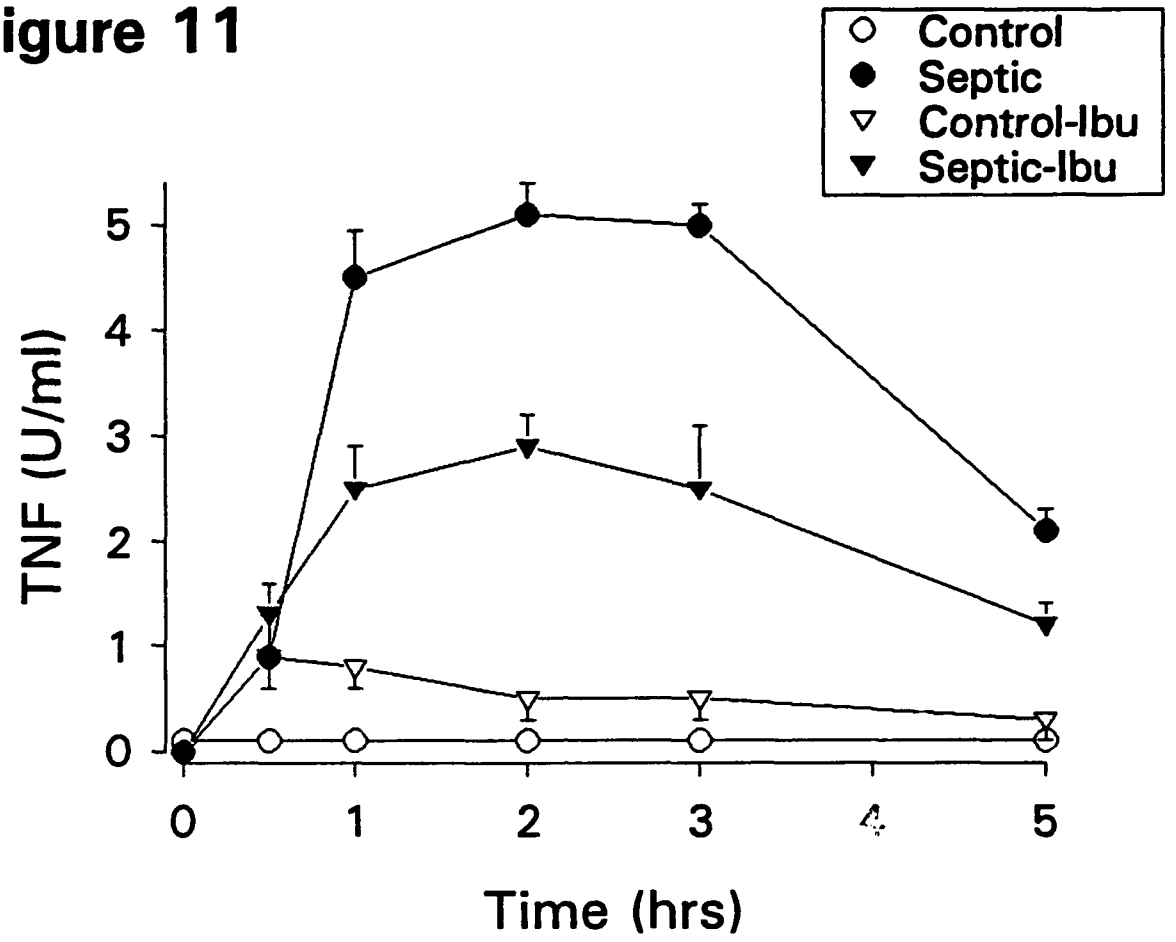
Bronchoalveolar Lavage Protein Content. Pseudomonas sepsis produced significant increases in alveolar protein content. IBU administration attenuated alveolar capillary membrane permeability to baseline values when administered as a pretreatment regimen. Delayed administration of IBU resulted in moderate protection when IBU was delayed by 30 minutes.

Figure 10



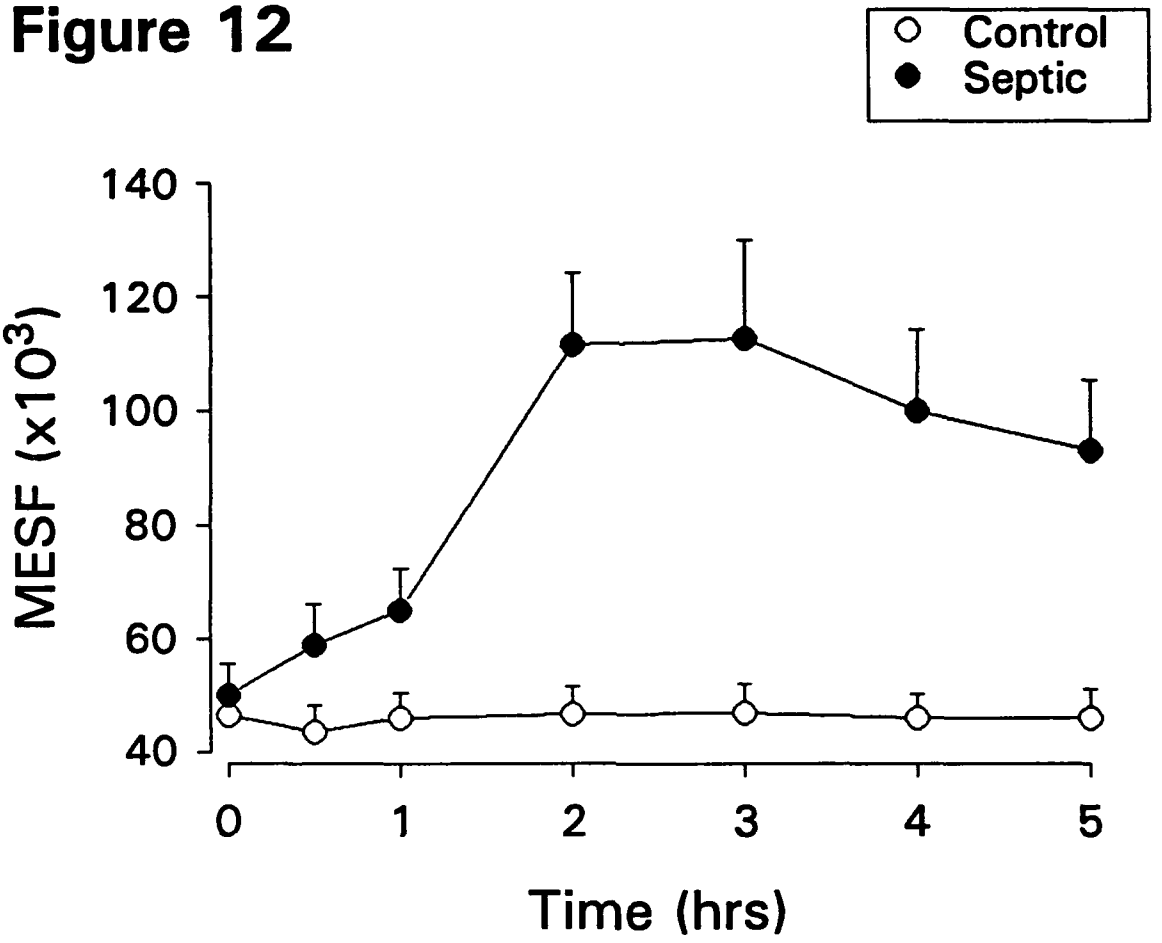
Peripheral Blood White Count (PMN). Septic animals exhibited rapid onset and profound neutropenia. IBU administration prior to onset of sepsis failed to maintain PMN in circulation.

Figure 11



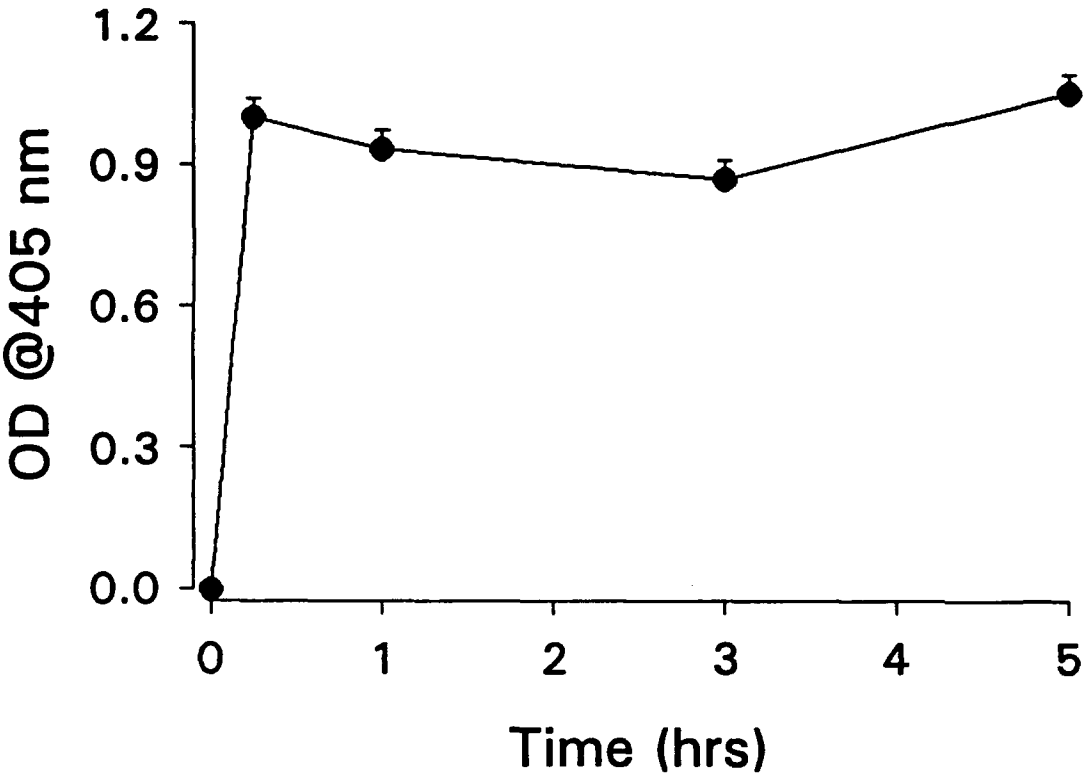
Tumor Necrosis Factor (TNF) Activity. Sepsis was associated with rapid onset of surging TNF activity in plasma. IBU administration resulted in attenuation of TNF activity in septic animals over the course of sepsis. IBU administered to control animals resulted in minor increases in plasma TNF activity.

Figure 12



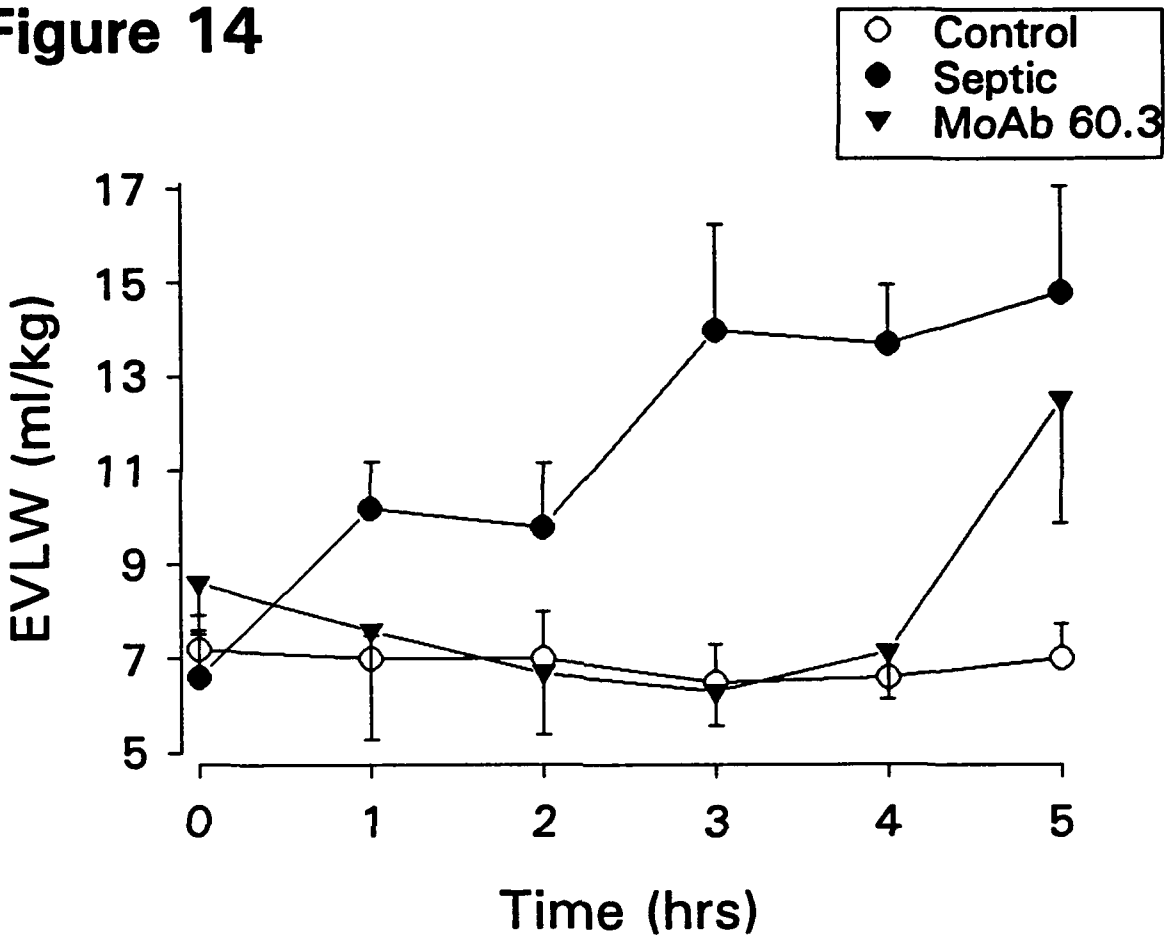
Blood Neutrophil CD11/CD18 Expression. Following onset of sepsis, neutrophil adhesion receptor expression surged peaking at 2 hours. PMN from control animals was unchanged over the period of observation.

Figure 13



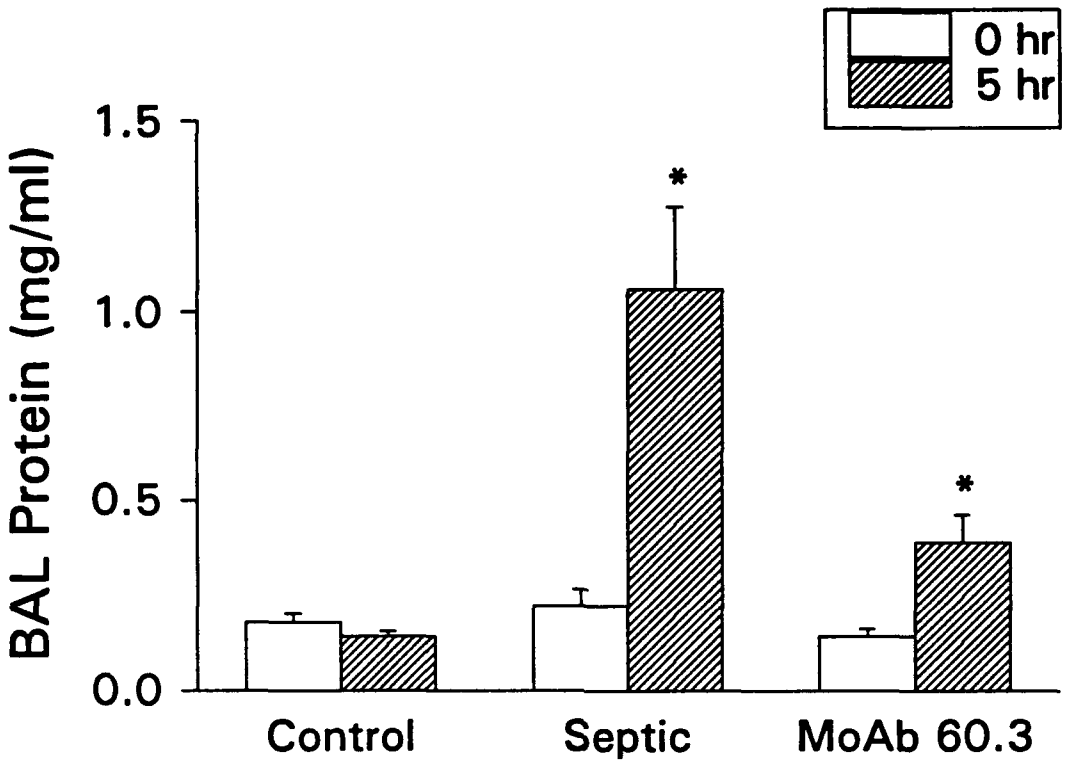
Serum Monoclonal Antibody 60.3 Levels. Following infusion of MoAb 60.3 serum levels increased significantly and stayed unchanged for 5 hours of study.

Figure 14



Extravascular Lung Water: Effects of MoAb 60.3. MoAb 60.3 administration significantly attenuated EVLW in septic animals. Towards the end of study EVLW levels rose to levels intermediate between septic and control

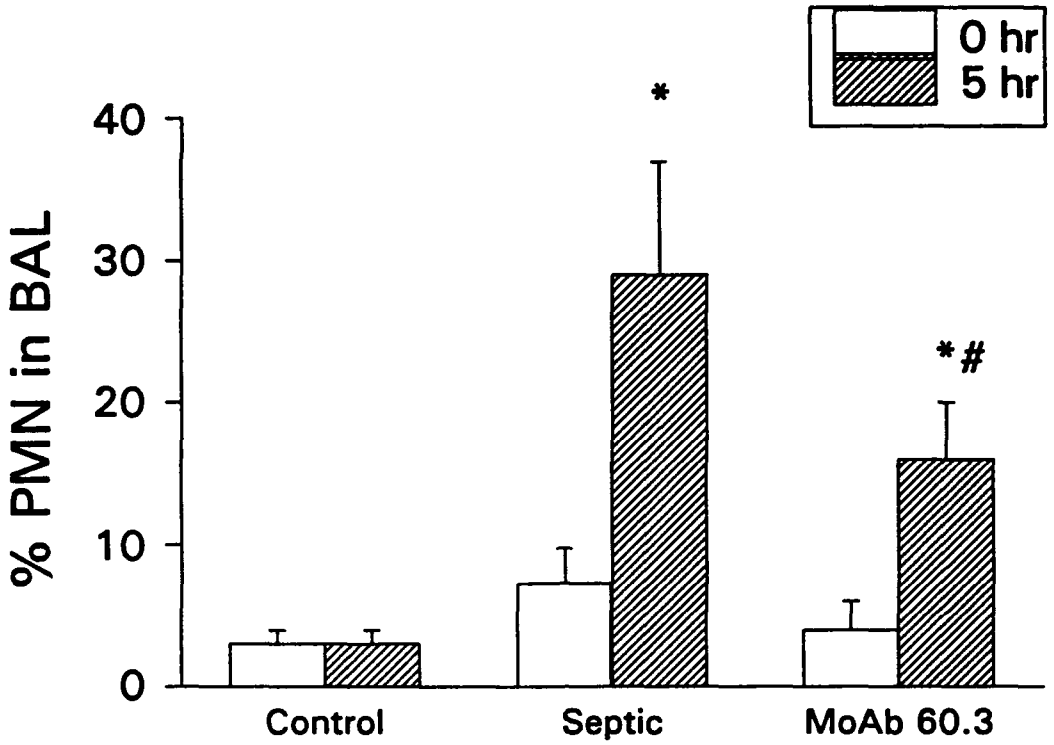
Figure 15



*p<0.05 vs 0 hr

Bronchoalvelar Lavage (BAL) Protein content: Effects of MoAb 60.3. Septic animals exhibited significant increases in BAL protein content following 5 hours of sepsis indicating increased alveolar capillary membrane permeability. MoAb 60.3 significantly reduced BAL protein content.

Figure 16

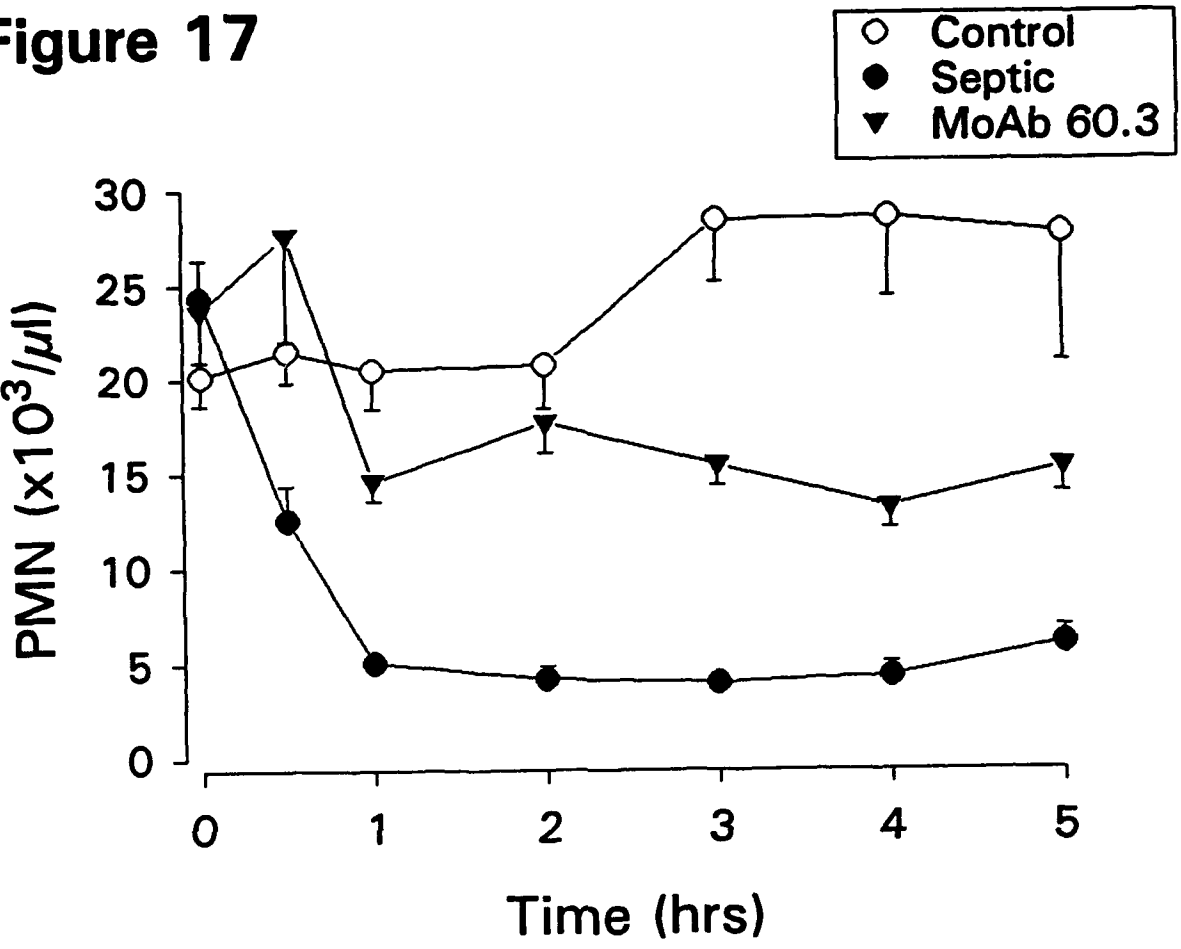


* $p<0.05$ vs O hr

# $p<0.05$ vs Septic 5 hr

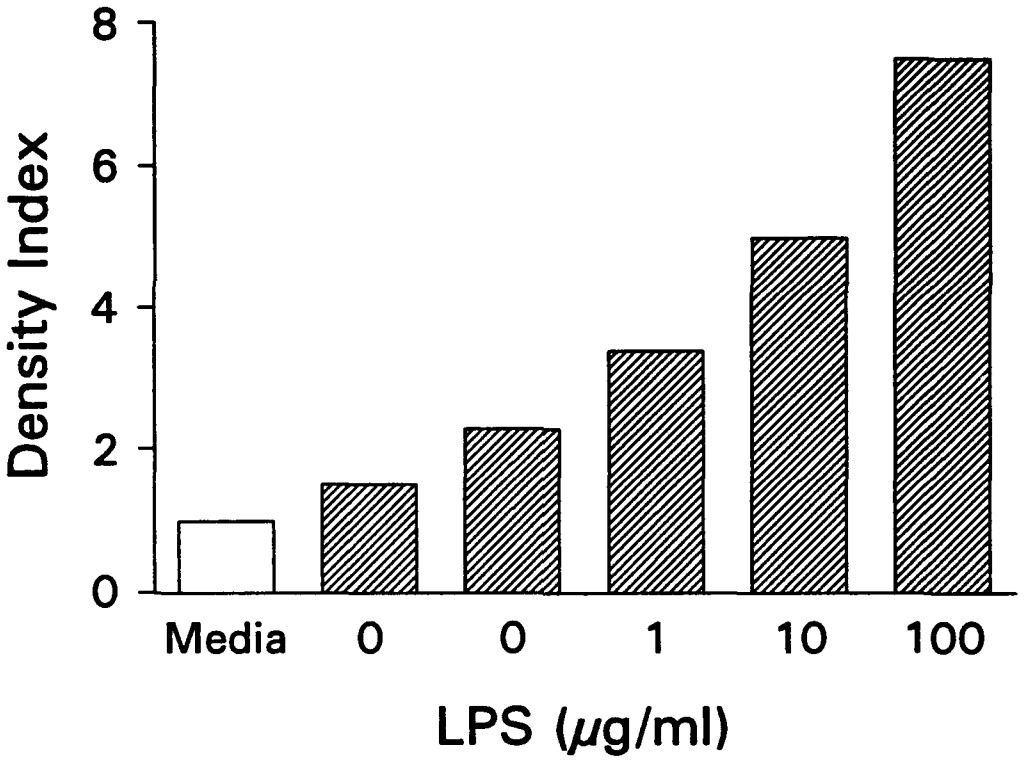
Bronchoalveolar Lavage Neutrophil (PMN). Septic animals exhibited a significant neutrophilic alveolitis at 5 hours of study. MoAb 60.3 markedly attenuated PMN migration into the airspaces over 5 hours of study.

Figure 17



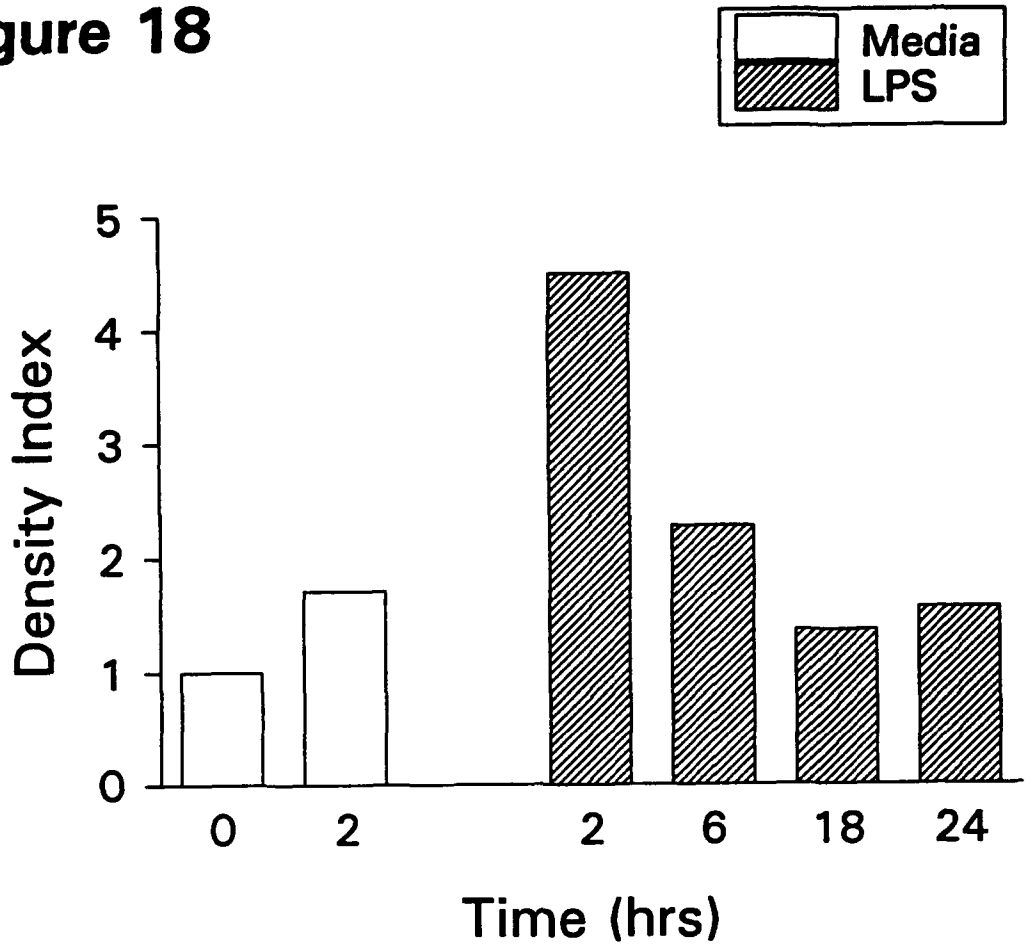
Circulating Blood Neutrophil (PMN) counts. Septic animals exhibited acute onset of severe neutropenia over the course of the septic process. MoAb 60.3 significantly attenuated the neutropenia stablizing blood PMN concentrations from 60 minutes to 300 min.

Figure 19



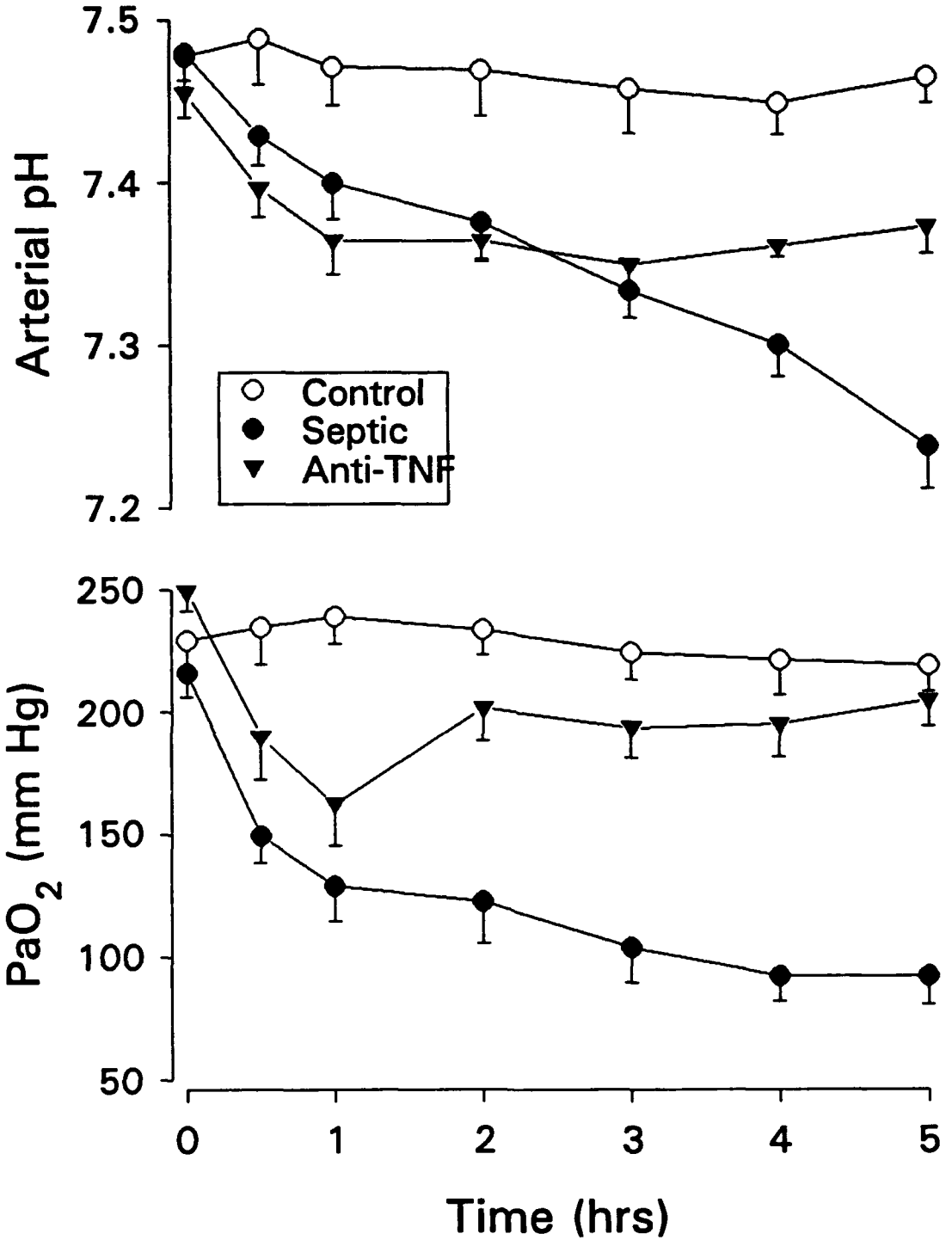
Steady State Transforming Growth Factor Beta levels in isolated Pulmonary Intravascular Macrophages exposed to increasing concentrations of endotoxin (LPS)

Figure 18



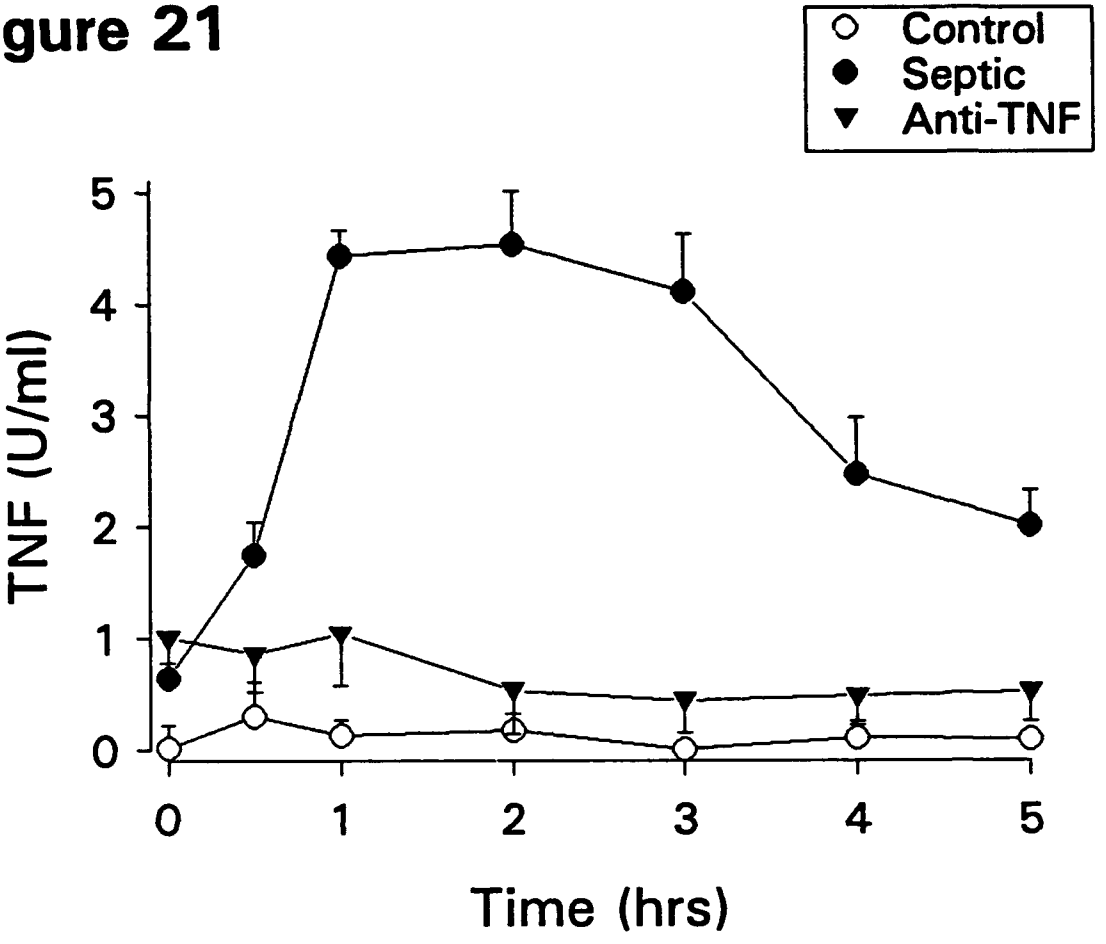
Steady state Transforming Growth Factor Beta mRNA levels present in isolated Pulmonary Intravascular Macrophages following exposure to 10 micrograms/ml of endotoxin.

Figure 20



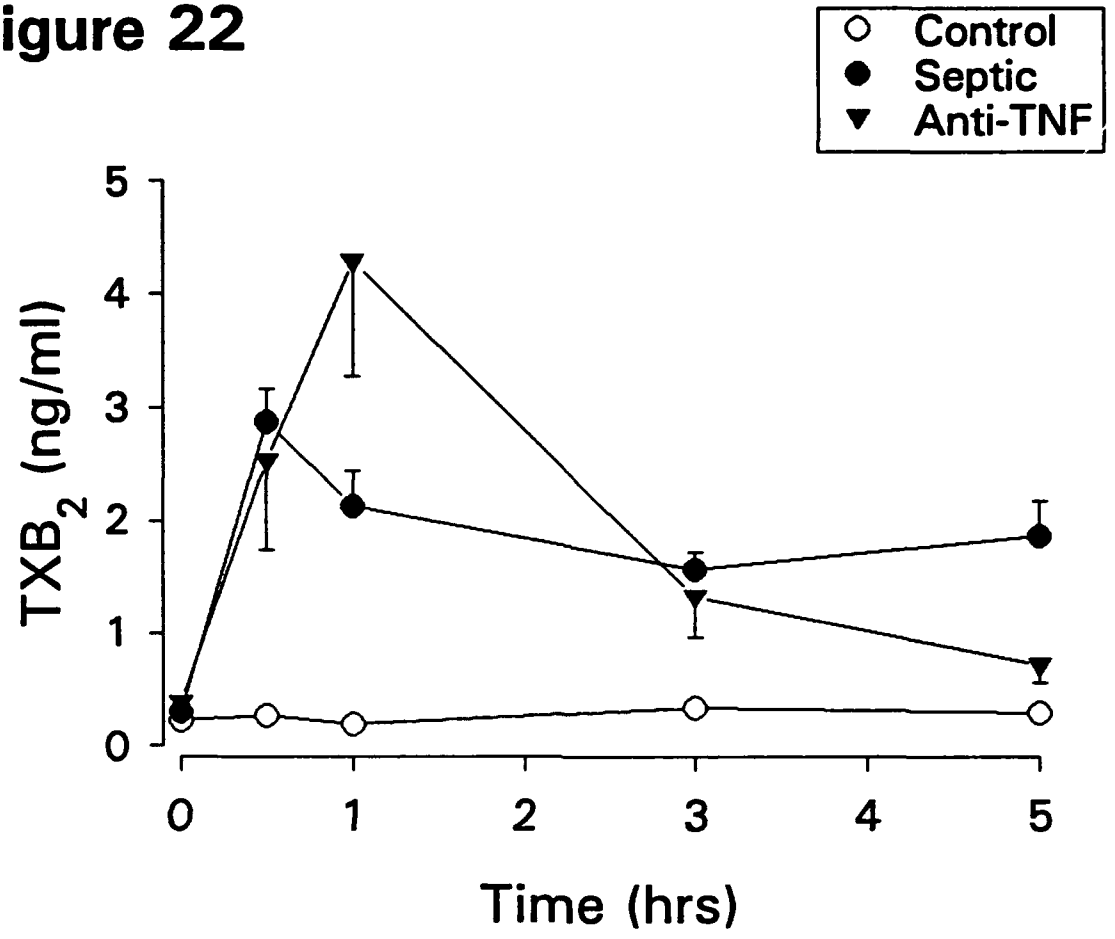
Effects of Anti-TNF Alpha MoAb on arterial blood pH and arterial oxygen tension following onset of sepsis. We found that pretreatment with antibody (5 mg/ml) prior to onset of sepsis produced improved acid base and oxygenation variables.

Figure 21



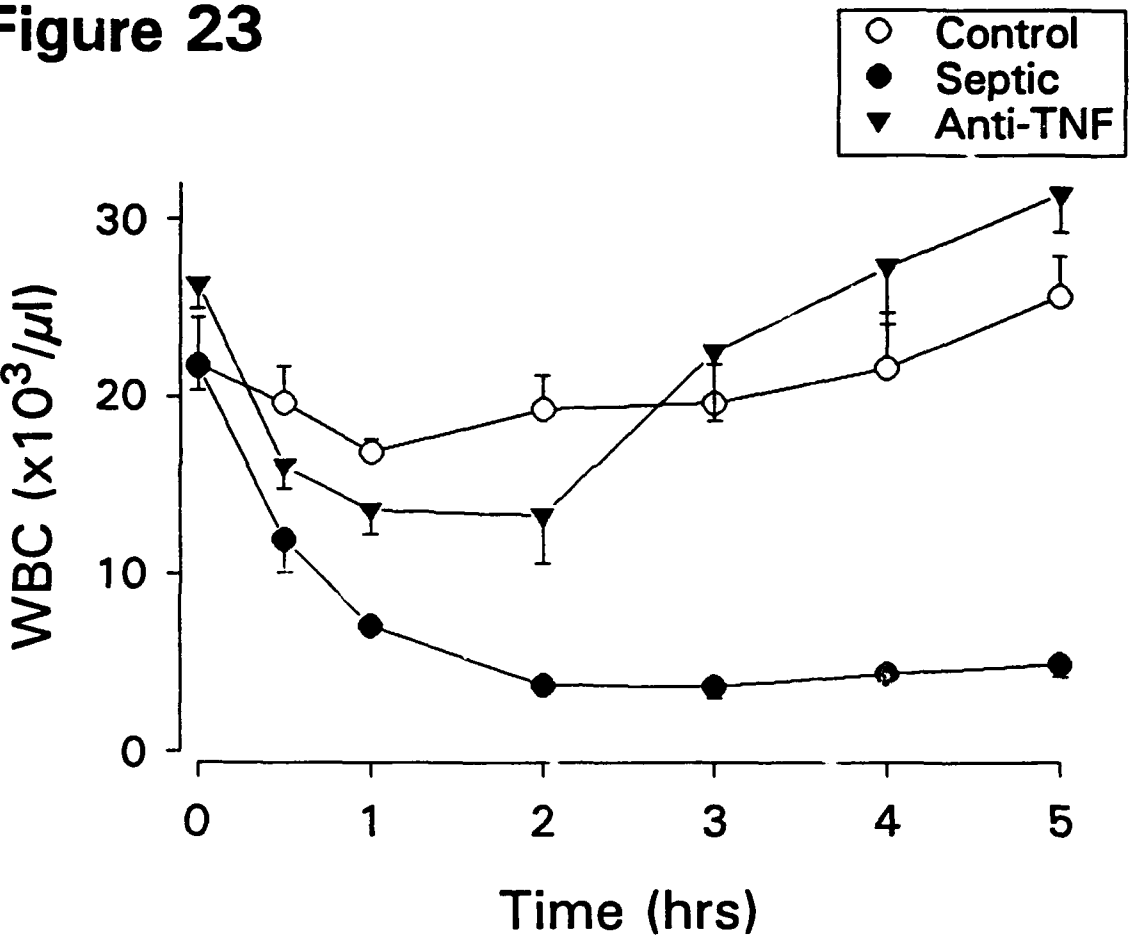
Plasma TNF Activity. Septic animals exhibited an early phase surge in plasma TNF activity. Anti-TNF antibody abolished plasma TNF activity.

Figure 22



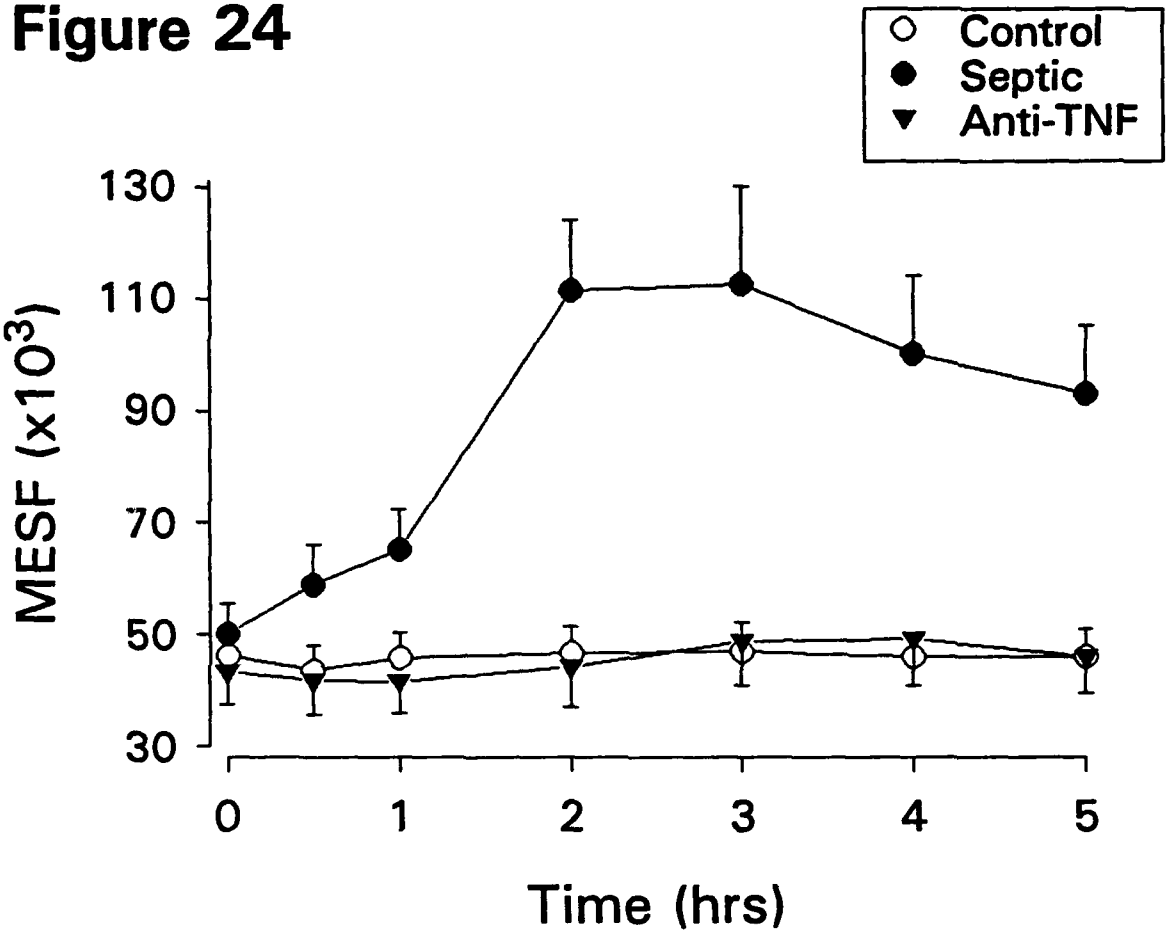
Plasma Thromboxane Levels. Septic animals exhibited surges in plasma thromboxane. Administration of Anti-TNF-alpha anitibody was associated with higher plasma levels peaking at 60 minutes.

Figure 23



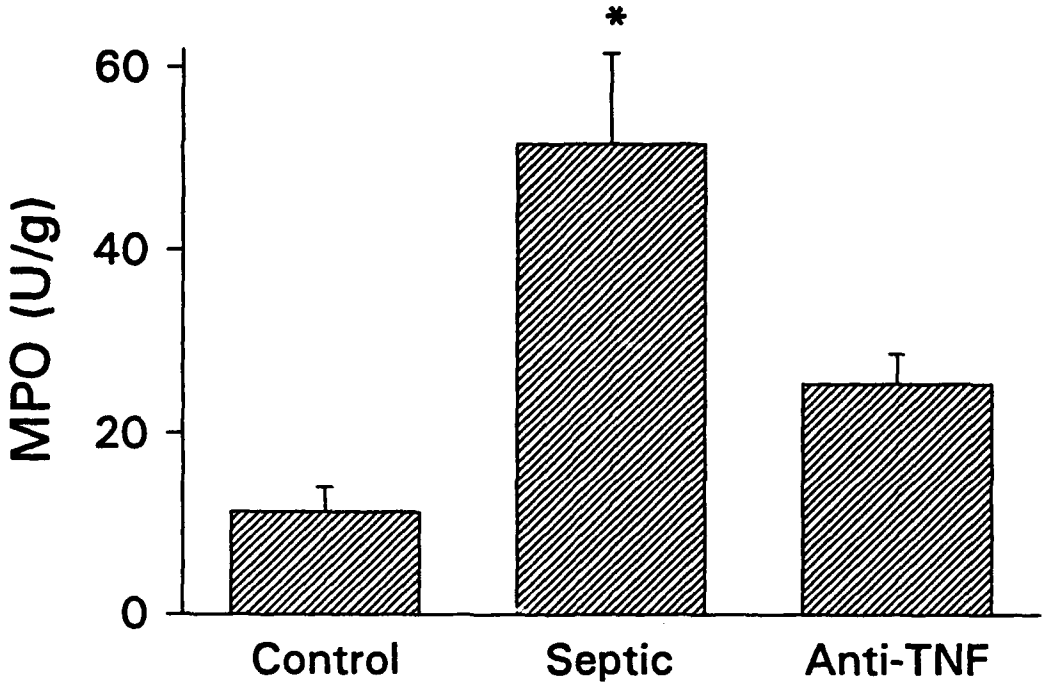
Circulating Neutrophil Counts (WBC) Following Anti-TNF-Alpha Administration. Septic animals pretreated prior to onset of Pseudomonas sepsis exhibited only a transient neutropenia reaching a nadir between 60 and 120 minutes. From 120 minutes to 300 minutes neutrophil counts re-bounded to levels not significantly different from saline controls.

Figure 24



Effects of Anti-TNF-Alpha Antibody On Neutrophil CD/11CD/18 expression. Septic animals pretreated with anti-TNF-Alpha showed no upregulation in neutrophil adhesion receptor expression over the course of sepsis.

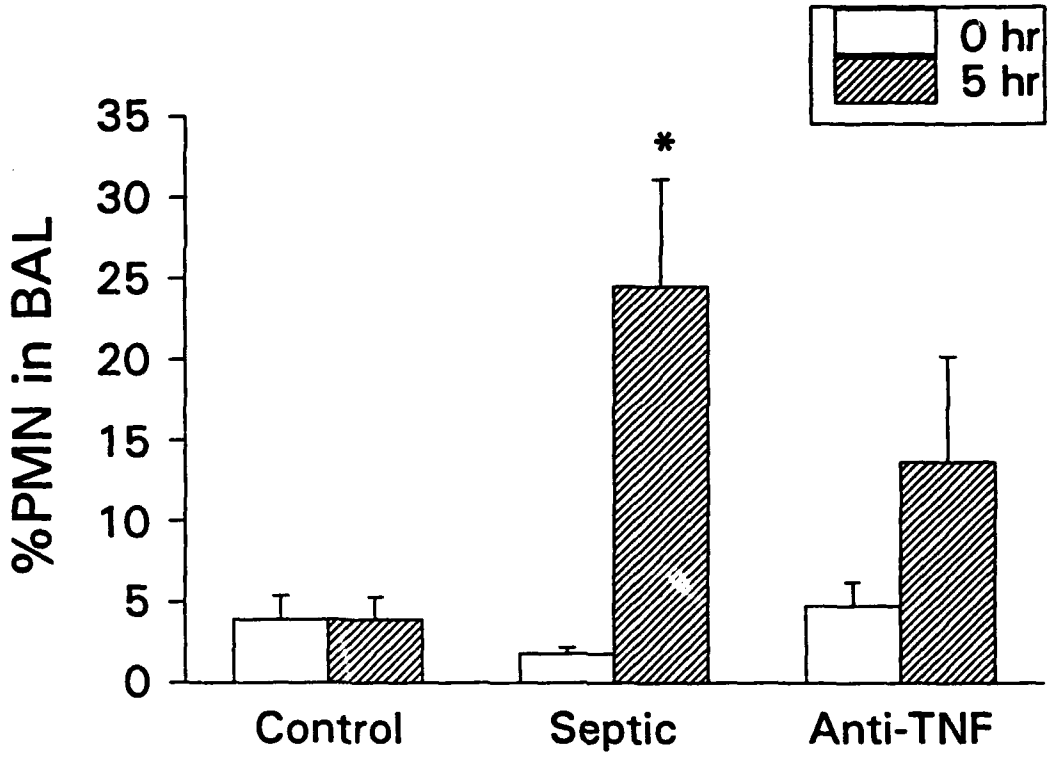
Figure 25



*p<0.05 vs Control

Whole Lung Homogenates Analyzed For Myeloperoxidase. Lung homogenates obtained from septic animals at 300 min showed significant increases in myeloperoxidase activity. Lungs obtained from animals pretreated with anti-TNF-alpha antibody showed significant reduction in activity indicating that neutrophil sequestration was significantly less in antibody treated animals.

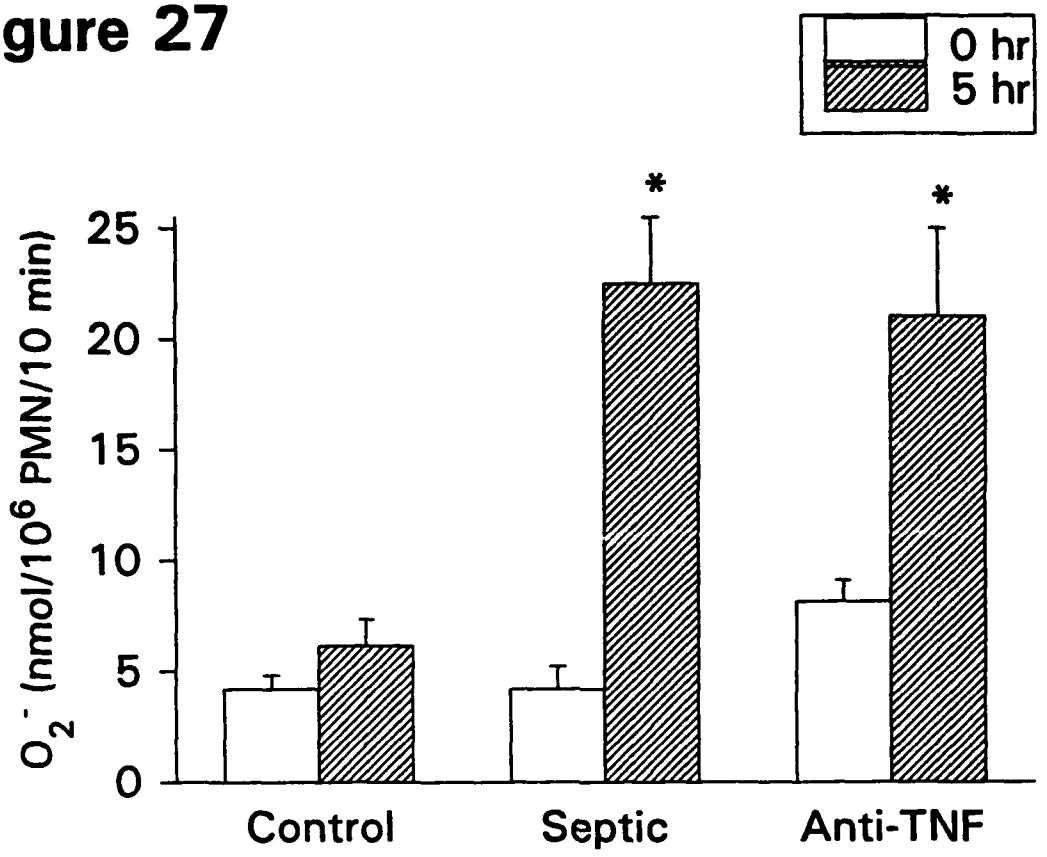
Figure 26



*p<0.05 vs Control

Bronchoalveolar Lavage Neutrophil Counts. Following onset of Pseudomonas sepsis, animals treated with Anti-TNF-alpha exhibited significant reduction in neutrophil migration into the alveolar spaces when compared to septic untreated animals.

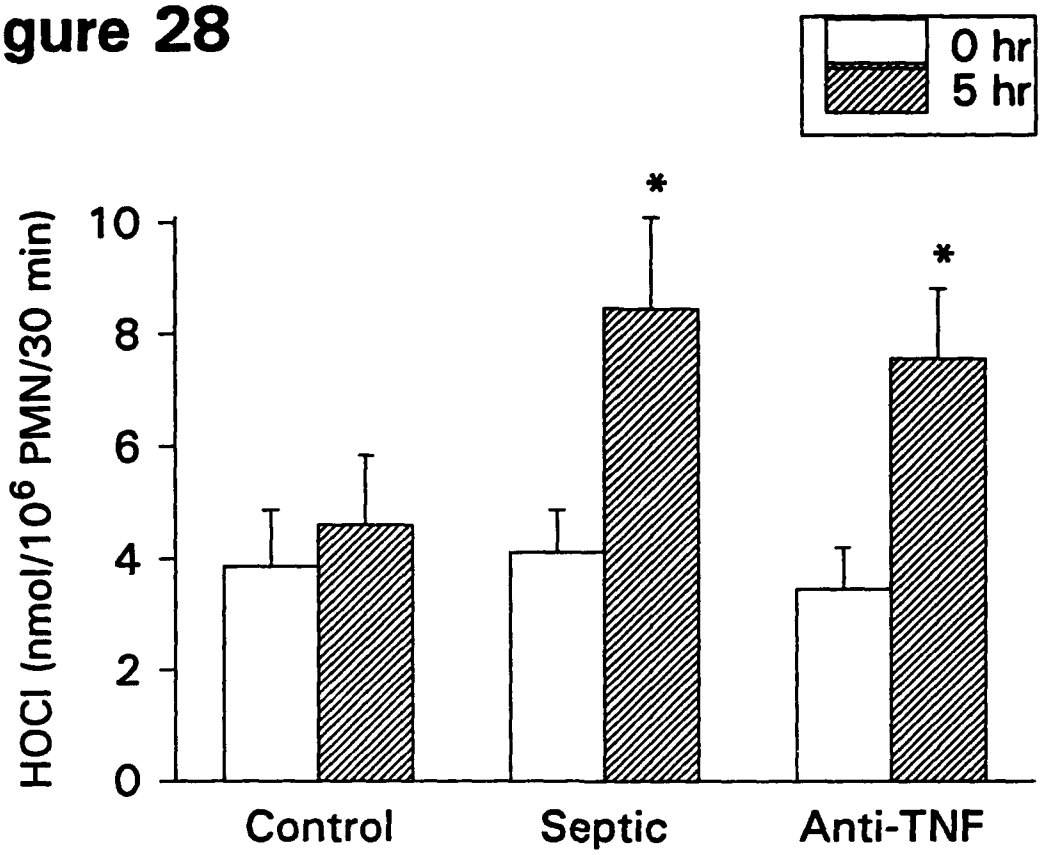
Figure 27



*$p<0.05$ vs Control

Neutrophil Superoxide Anion Production. Blood neutrophils from animals with untreated sepsis and pretreated with Anti-TNF-alpha exhibited the same degree of priming at 5 hours for superoxide anion generation.

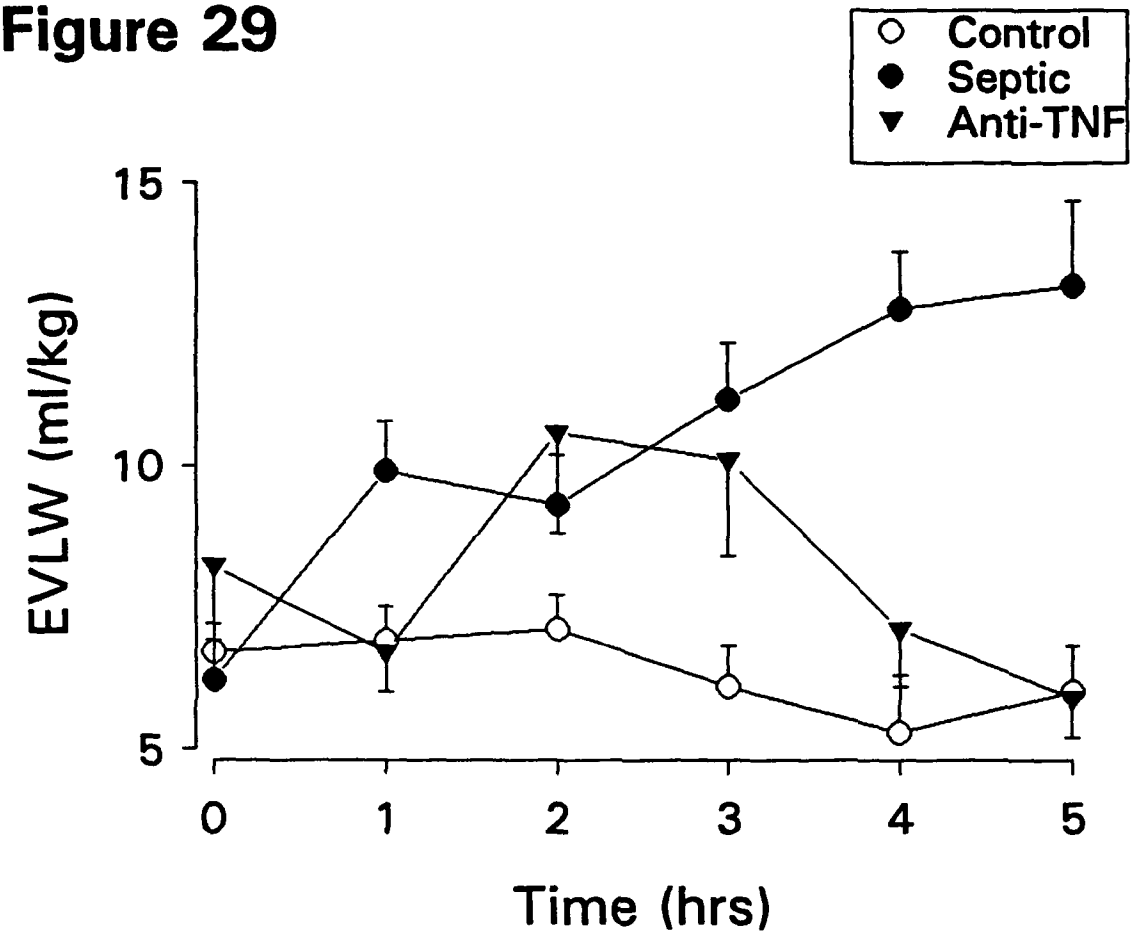
Figure 28



*p<0.005 vs 0 hr

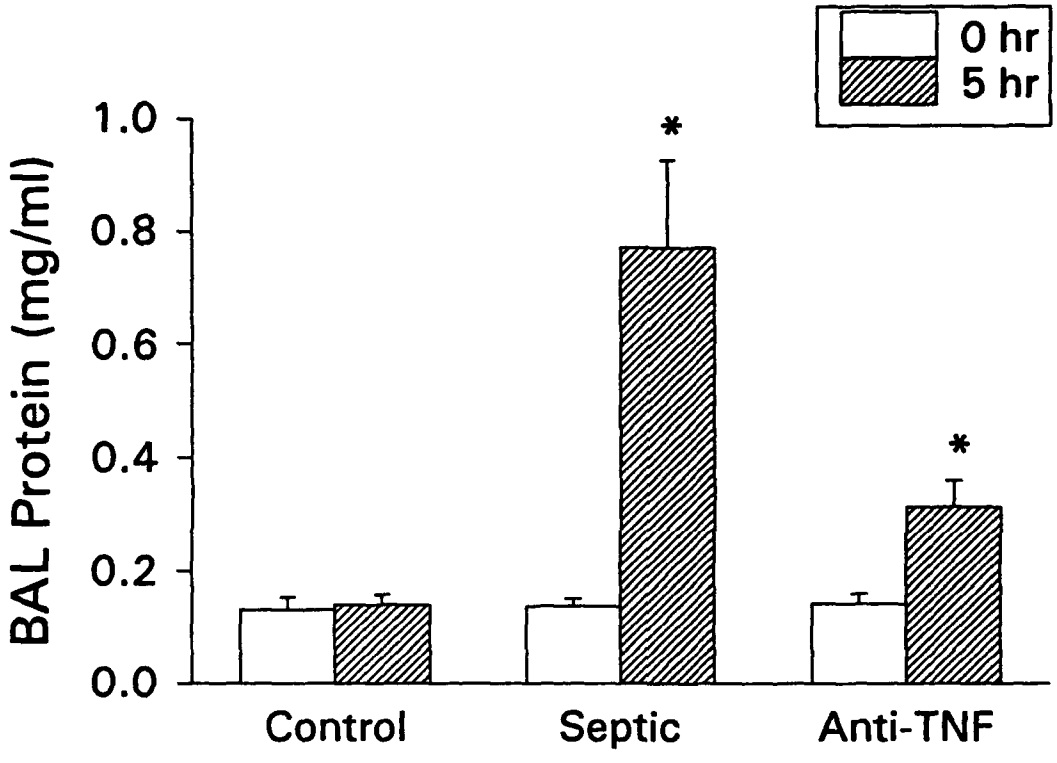
Neutrophil Hypochlorous Acid (HOCL) Production. Blood neutrophils obtained from both septic untreated animals and animals treated with anti-TNF-alpha antibody exhibited identical degrees of priming for enhanced HOCL generation following 5 hours of sepsis.

Figure 29



Extravascular Lung Water. Animals pretreated with anti-TNF-alpha antibody exhibited increases in EVLW reaching a peak at 120 minutes then returning to baseline by five hours of sepsis

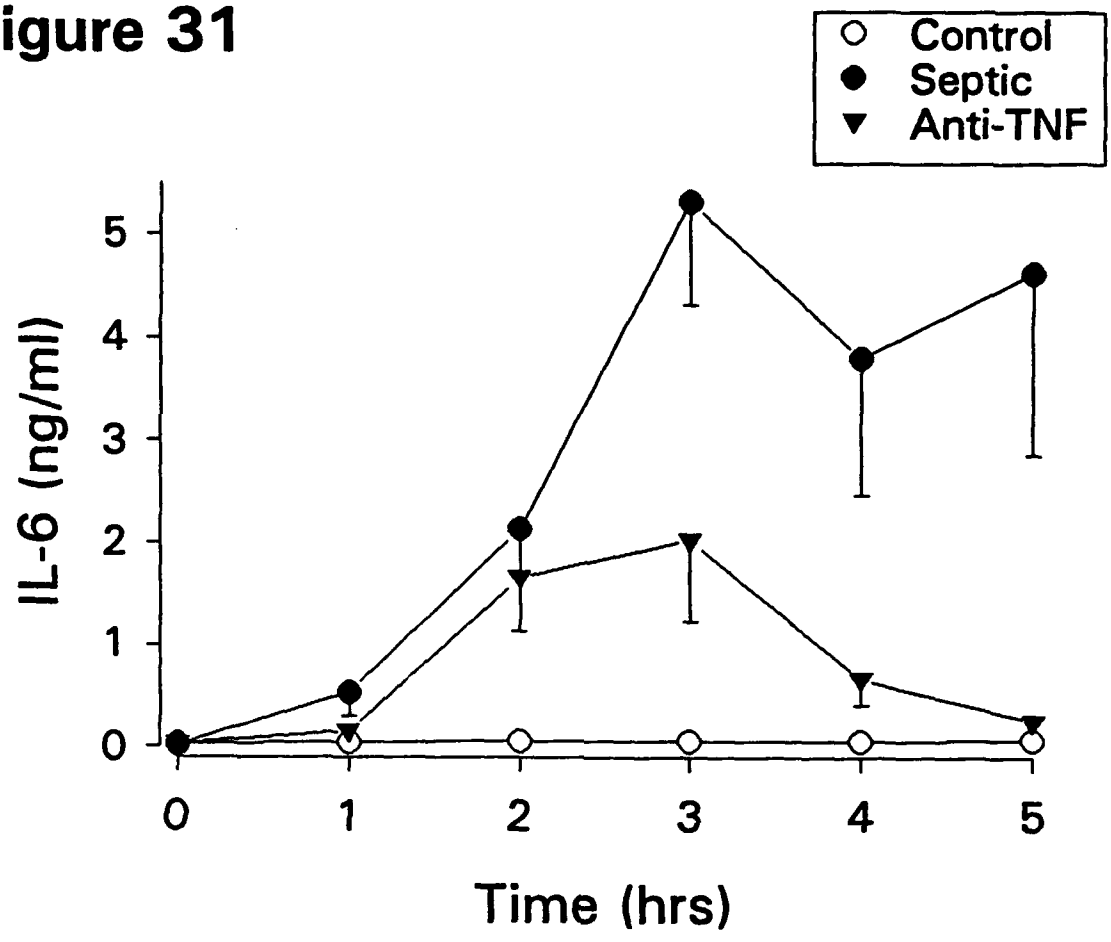
Figure 30



*$p < 0.05$ vs 0 hr

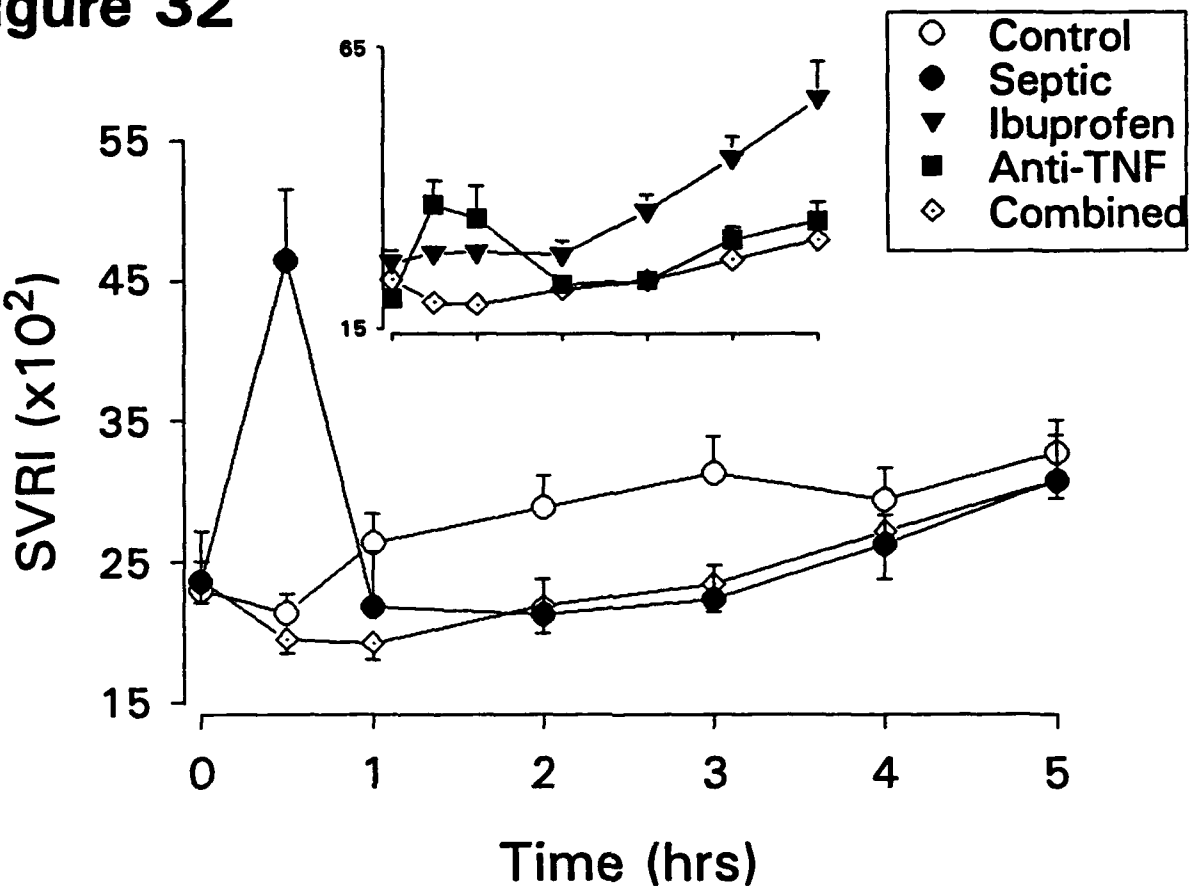
Bronchoalveolar Lavage Protein Content. The degree of alveolar capillary membrane permeability as assessed by bronchoalveolar lavage protein content was significantly less at 5 hours of sepsis in anti-TNF-alpha antibody treated animals.

Figure 31



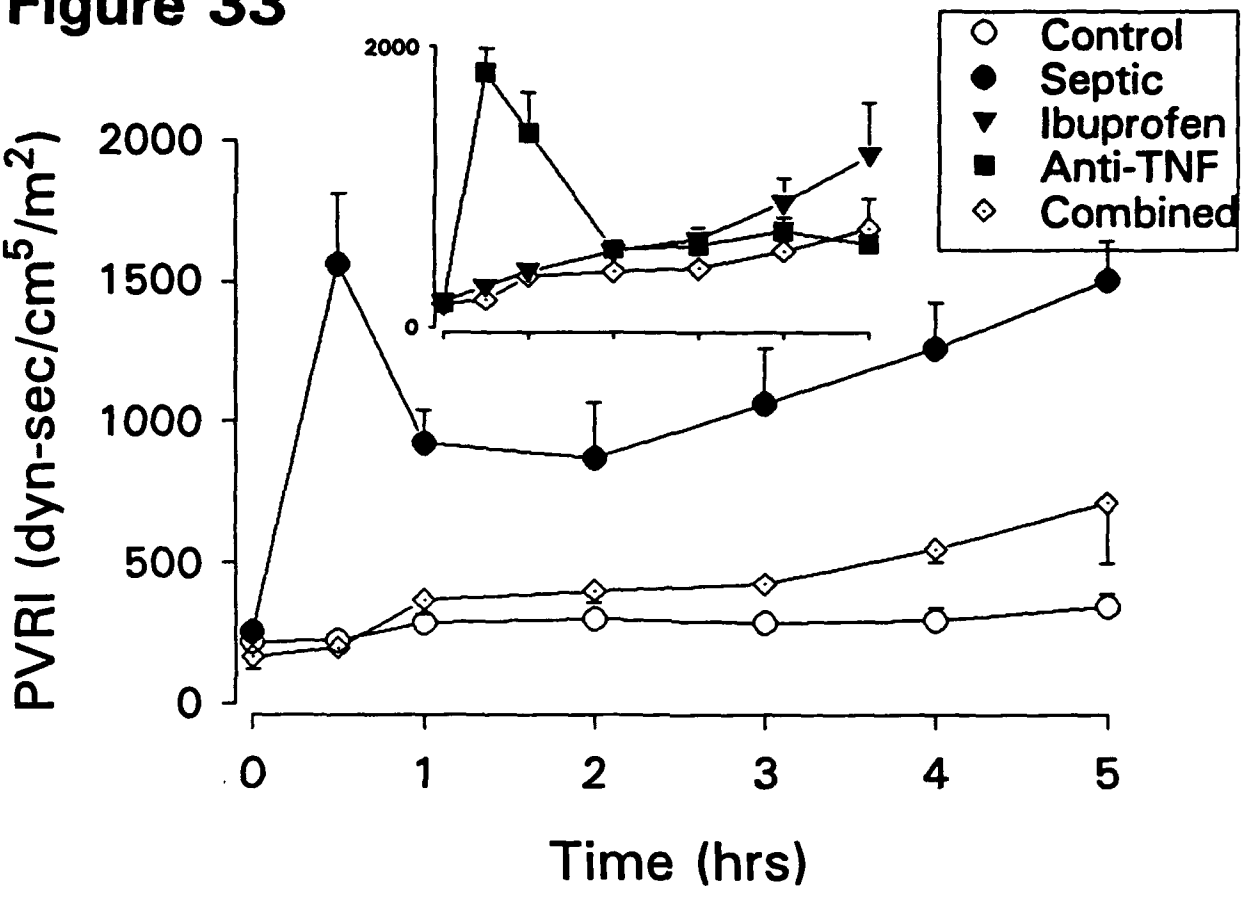
Plasma Interleukin-6 Levels: The Effects of Anti-TNF-alpha. Animals pretreated with 5 mg/kg anti-TNF-alpha antibody showed a markedly attenuated surge of IL-6 levels in plasma over the course of 5 hours of sepsis.

Figure 32



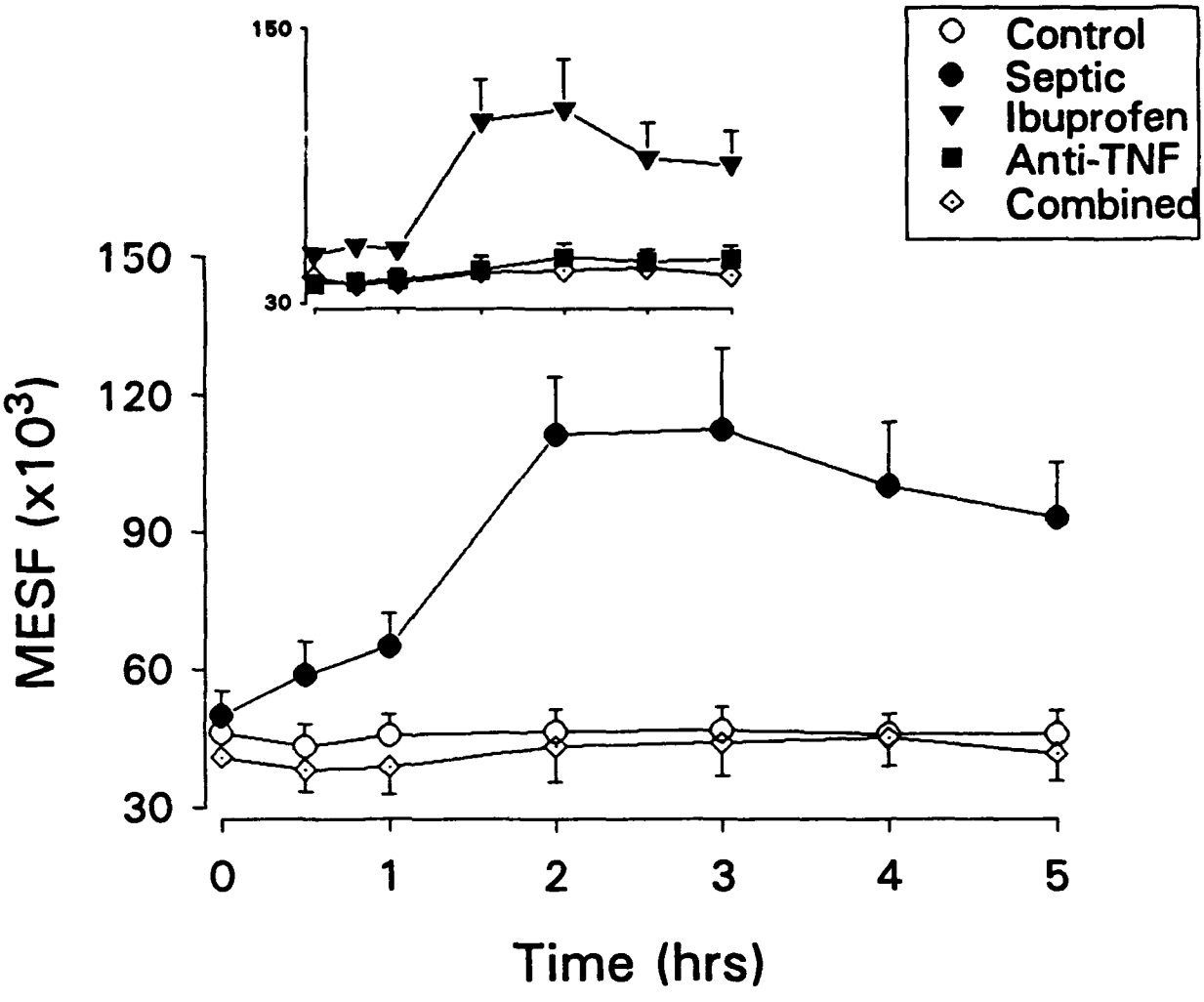
Systemic Vascular Resistance Index. Septic animals exhibited biphasic SVRI with an initial rise at 30 minutes followed by a gradual increase to the 5 hour time point. Combined agents (Ibuprofen, 12 mg/kg, Anti-TNF-alpha, 5 mg/kg) attenuated initial phasic septic increases but failed to attenuate subsequent late phase SVRI increases. IBU alone attenuated early phase SVRI increases, Anti-TNF-alpha alone attenuated late phase increases.

Figure 33



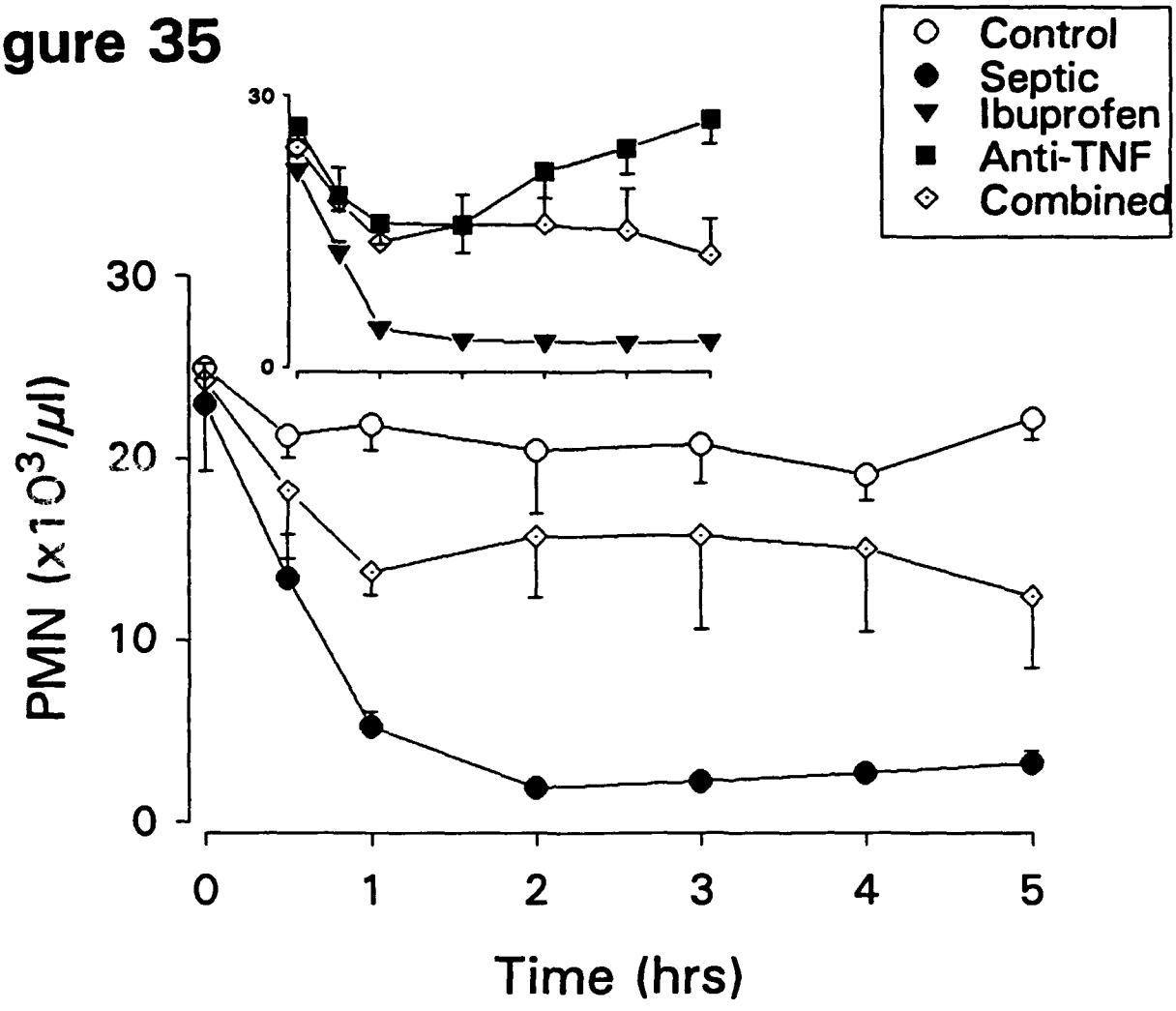
Pulmonary Vascular Resistance Index. Septic animals exhibited sharp increases in PVRI in early phase sepsis followed by continual increases until 5 hours. Combined agents abolished both early and late phase increases in PVRI

Figure 34



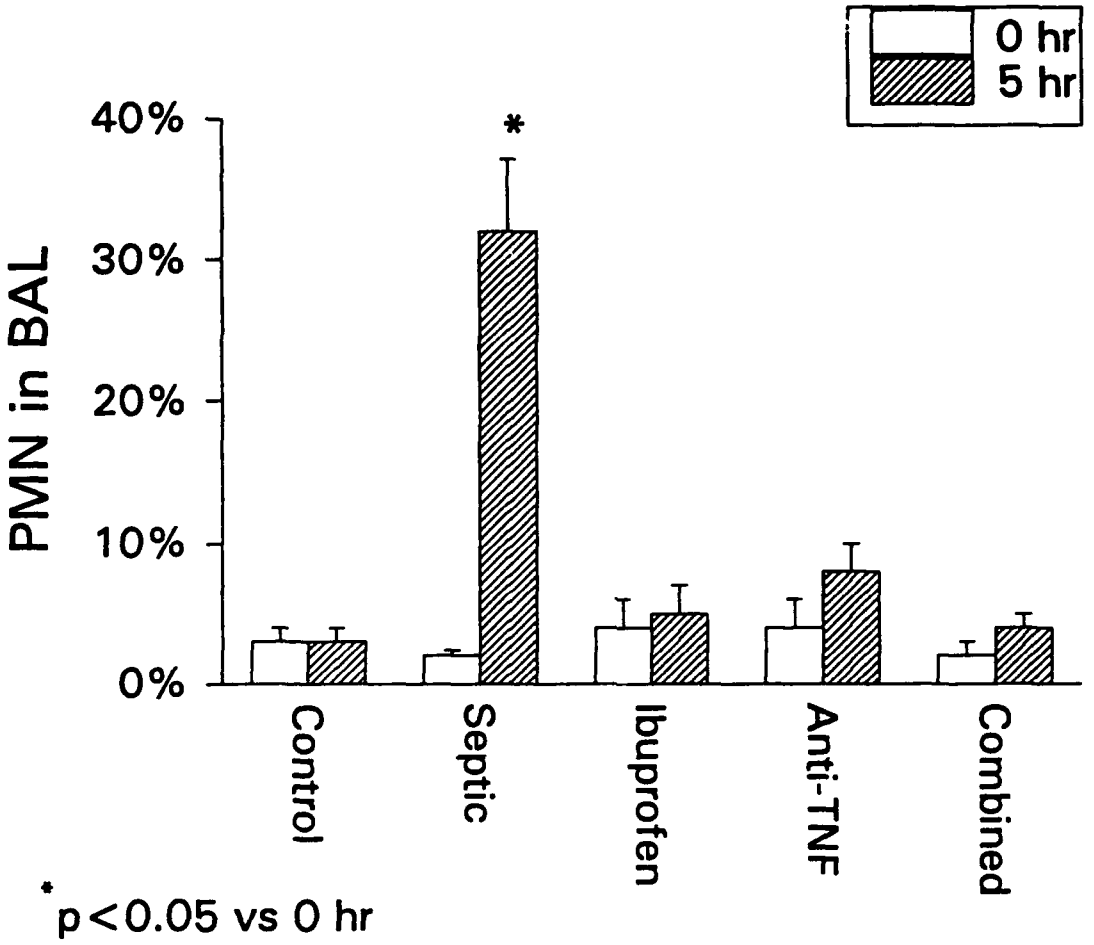
Effects Of Combined Agent Administration On Neutrophil. Adhesive Function In Sepsis. Combined agents administered exhibited identical capacity at attenuating the upregulation of CD11/CD18 in septic neutrophils. Combined agents exerted the same level of attenuating effects on Neutrophil CD11/CD18 expression as that observed in Anti-TNF-alpha alone. IBU alone failed to attenuate neutrophil adhesive response tosepsis.

Figure 35



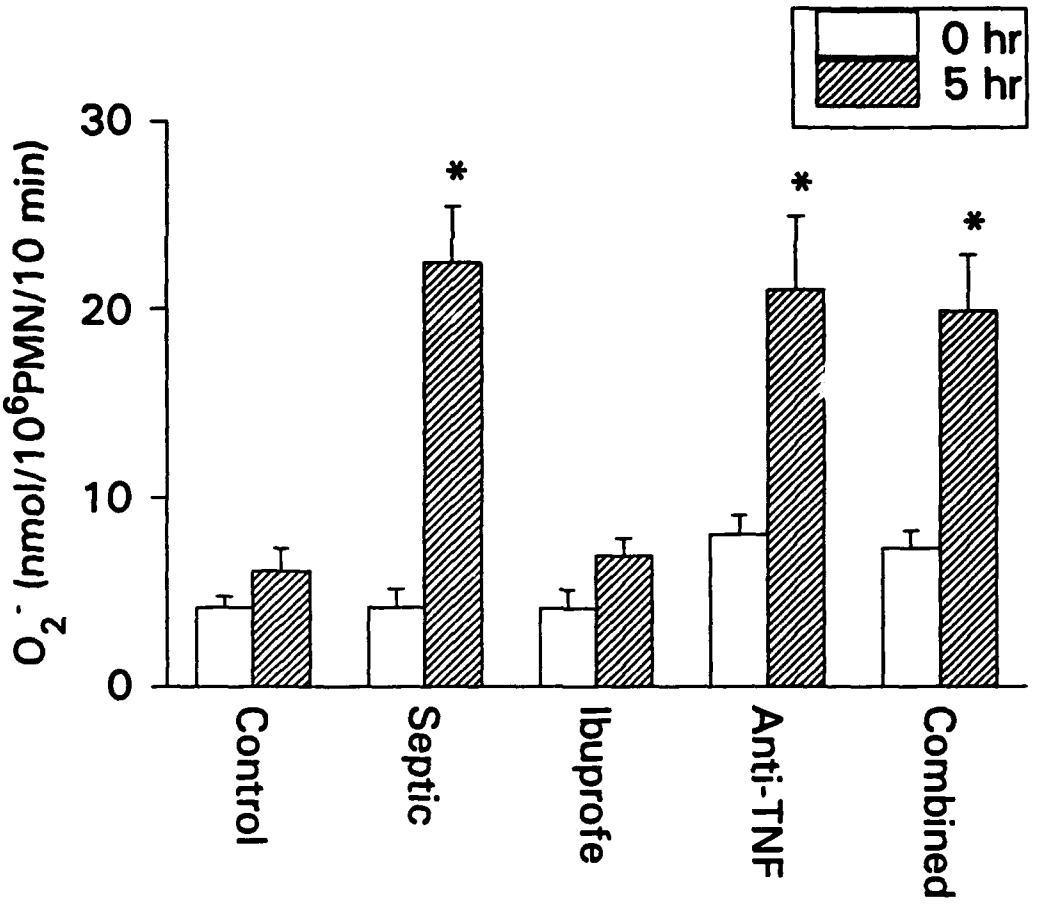
Effects of Combined Agents On Circulating Neutrophil Counts (PMN). The combination of IBU and Anti-TNF-alpha prevented the severe neutropenia associated with sepsis but was clearly different from anti-TNF-alpha alone. Combined agents prevented the invariable rebound neutrophilia associated with anti-TNF-alpha alone.

Figure 36



Bronchoalveolar Lavage Neutrophil Counts. When compared to septic untreated animals all agents tested (IBU *alone*, Anti-TNF-alpha *alone*, *combined* agents) were capable of preventing transvascular migration of neutrophils into the airspaces of the lung.

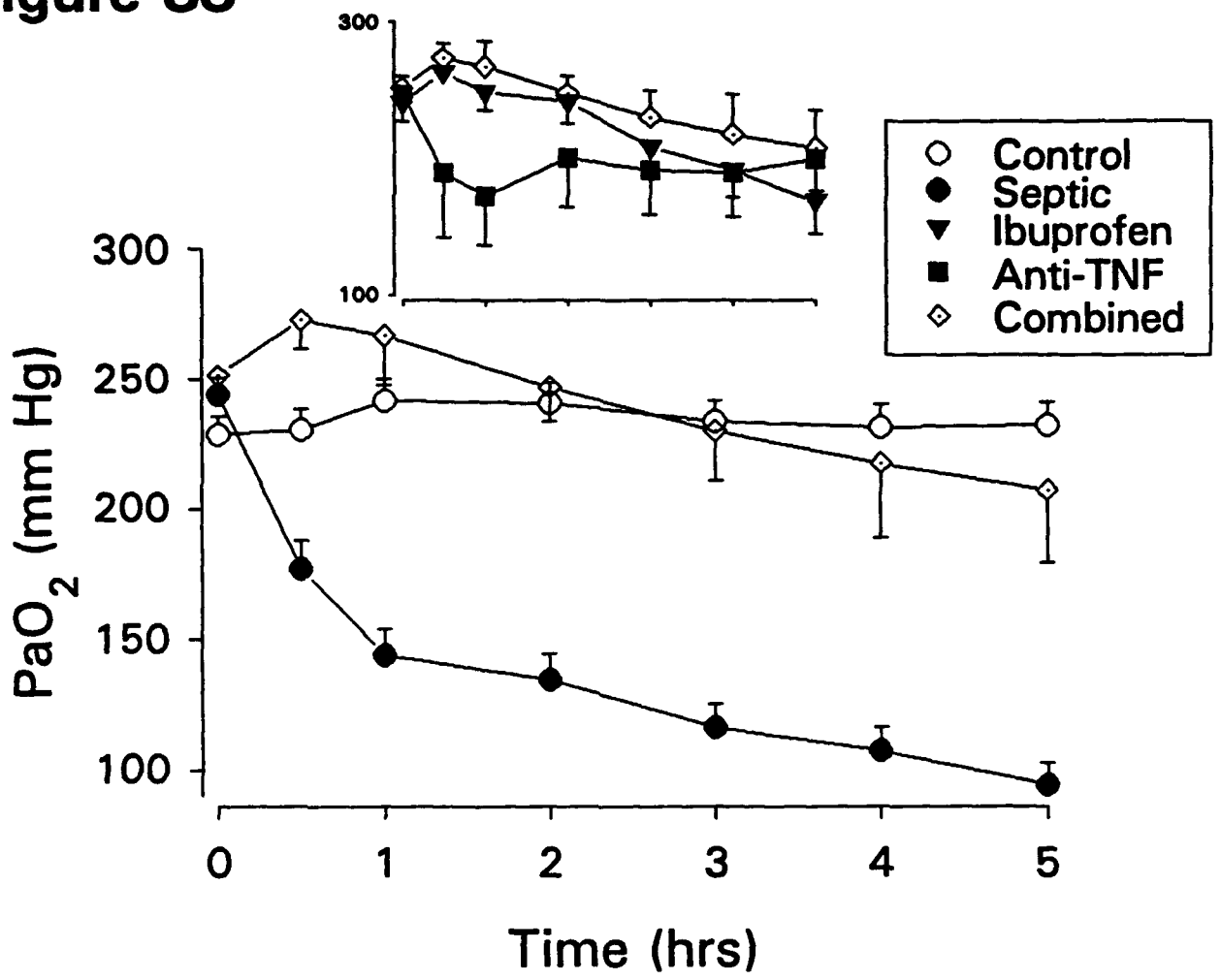
Figure 37



*p<0.05 vs 0 hr

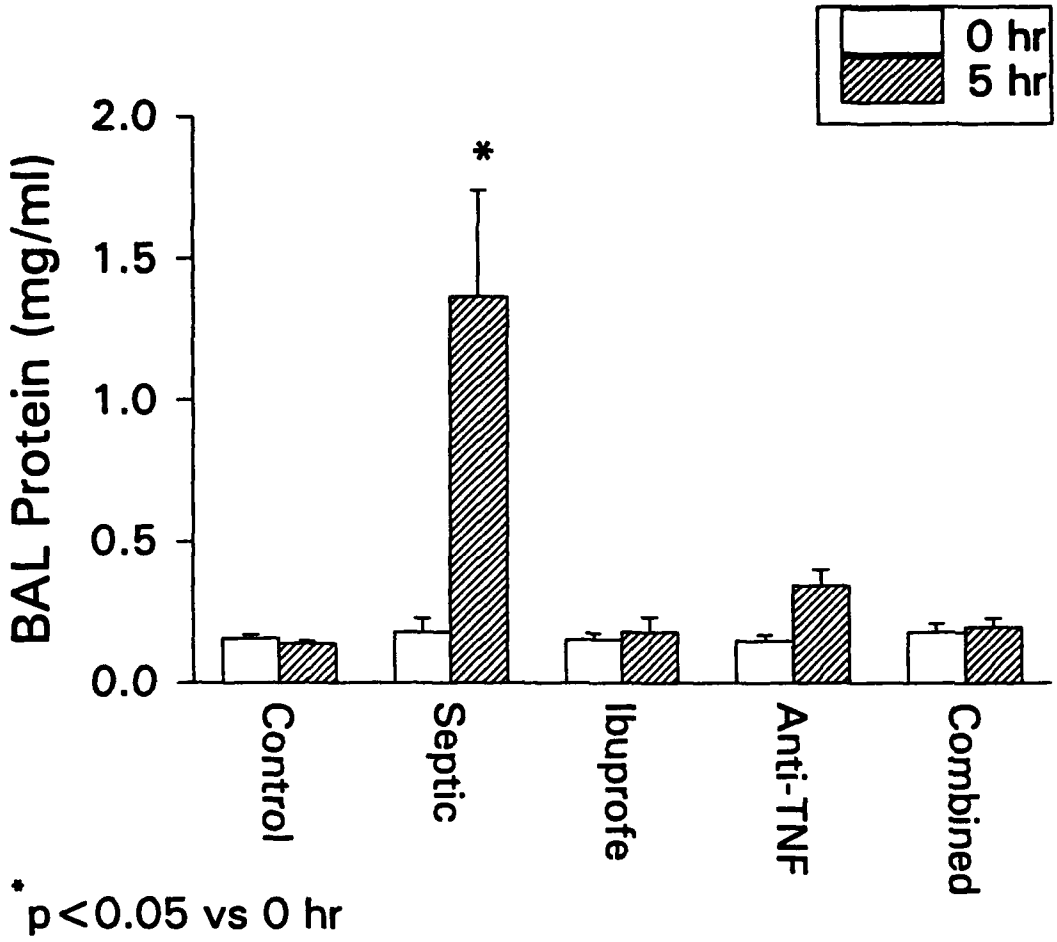
Neutrophil Superoxide Anion Generation. IBU administered to septic animals significantly downregulated PMN superoxide generation. Anti-TNF-alpha administered as a single agent failed to suppress PMN superoxide. When combined with anti-TNF anibody IBU lost its ability to suppress neutrophil oxidant burst.

Figure 38



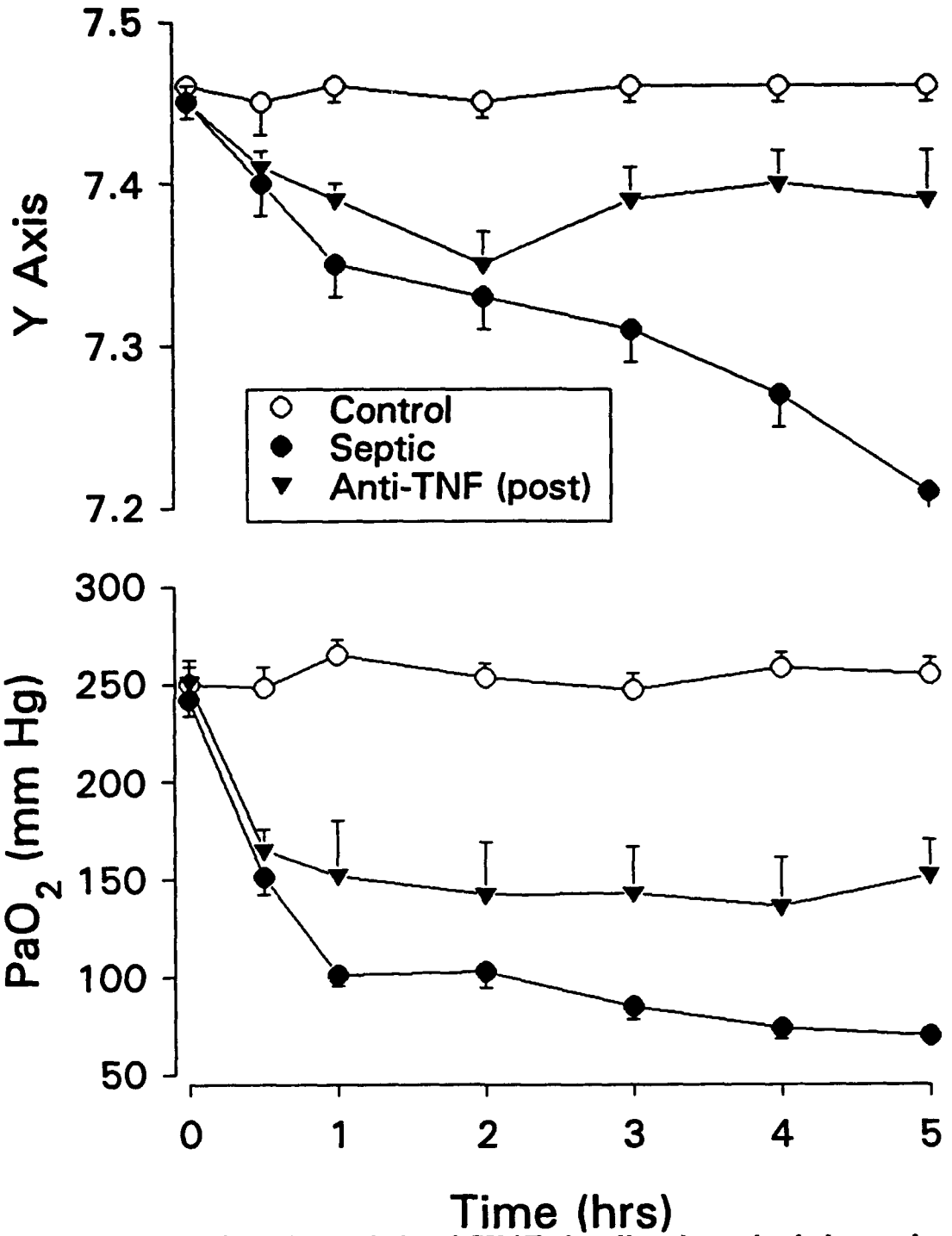
Blood Oxygen Tension. Combined agents maintained PO2 at control levels throughout the 5 hours of study. The effects of either agent alone are shown in the upper panel.

Figure 39



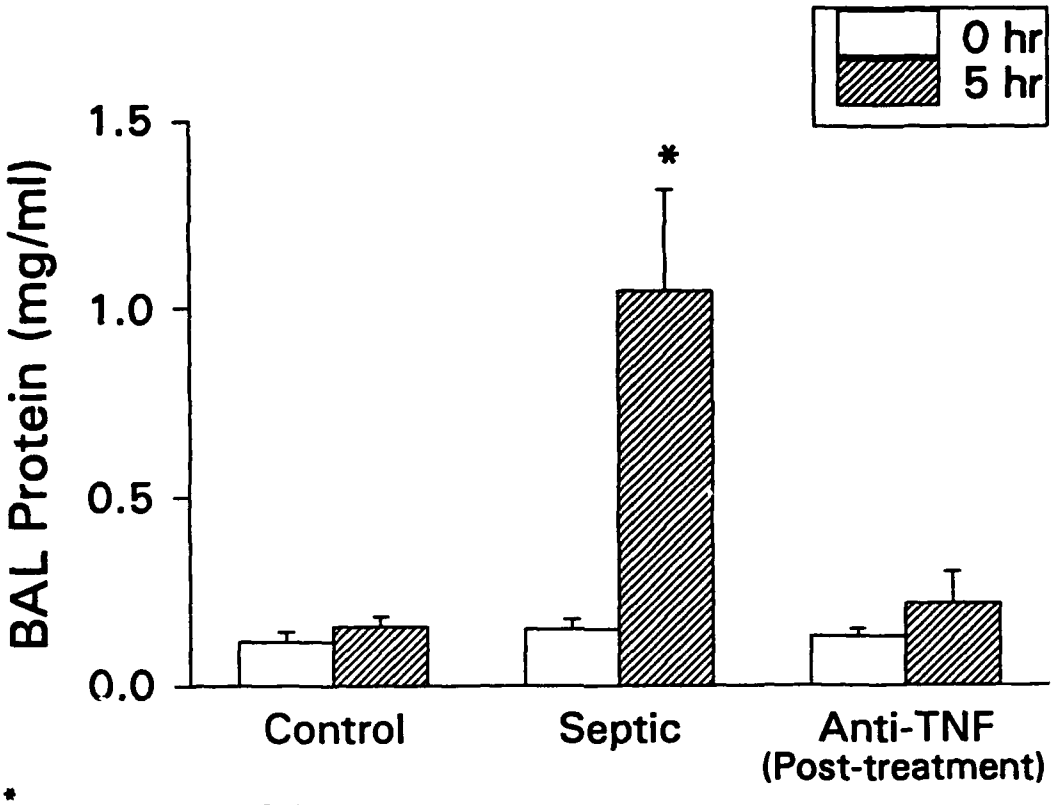
Bronchoalveolar Lavage Protein Content. Administration of combined agents or IBU alone or anti-TNF alone exhibited identical abilities to attenuate alveolar capillary membrane permeability associated with sepsis.

Figure 40



Effects of Delayed Anti-TNF Antibody administration In Sepsis. Delaying antibody administration until 60 minutes resulted in an initial drop in both arterial pH and PaO2. Blood pH recovered to near normal values and arterial PO2 was maintained at a stable level following administration until the end of study.

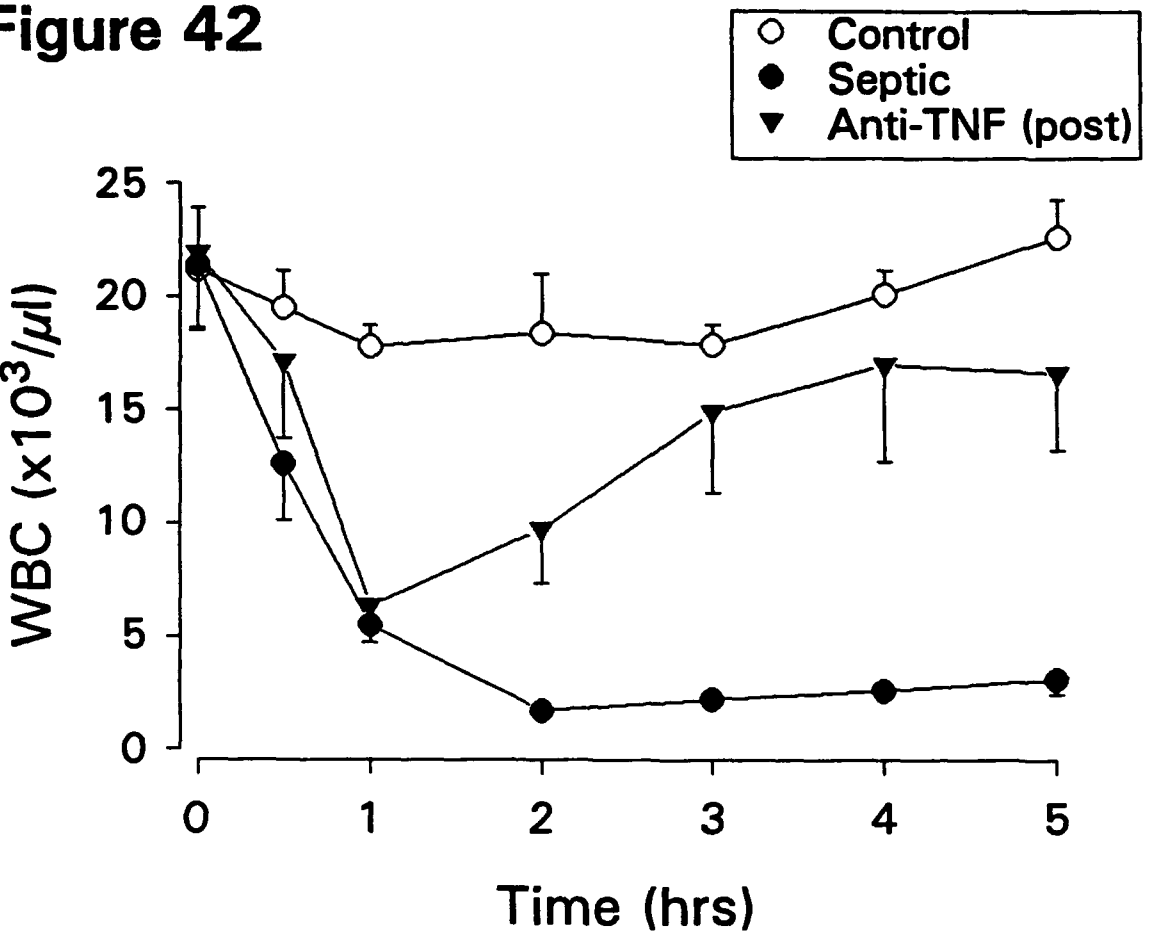
Figure 41



*p<0.05 vs 0 hr

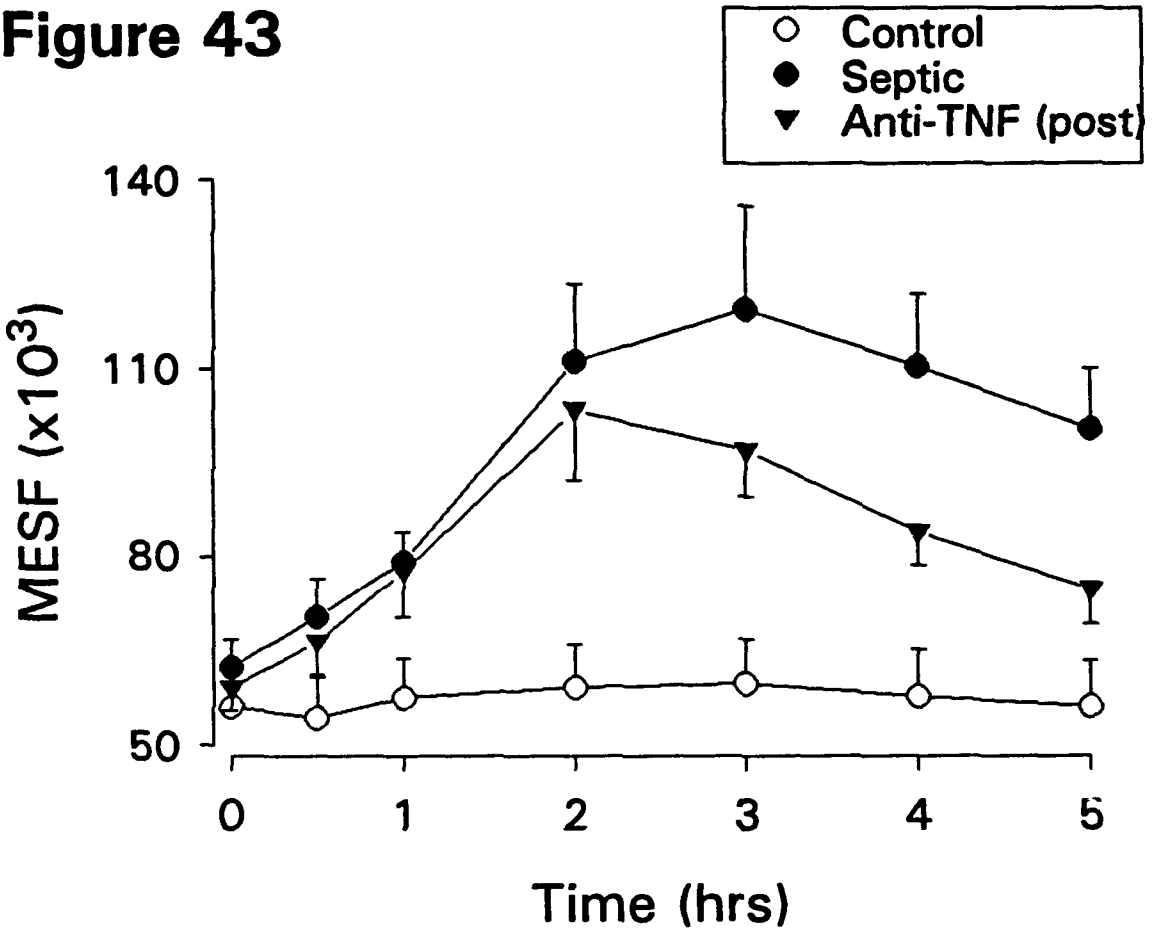
Effects Of Delayed Anti-TNF Antibody On Broncho-alveolar Lavage Protein. Administration of anti-TNF antibody 60 minutes following onset of sepsis resulted in significant attenuation of the alveolar capillary membrane permeability associated with sepsis.

Figure 42



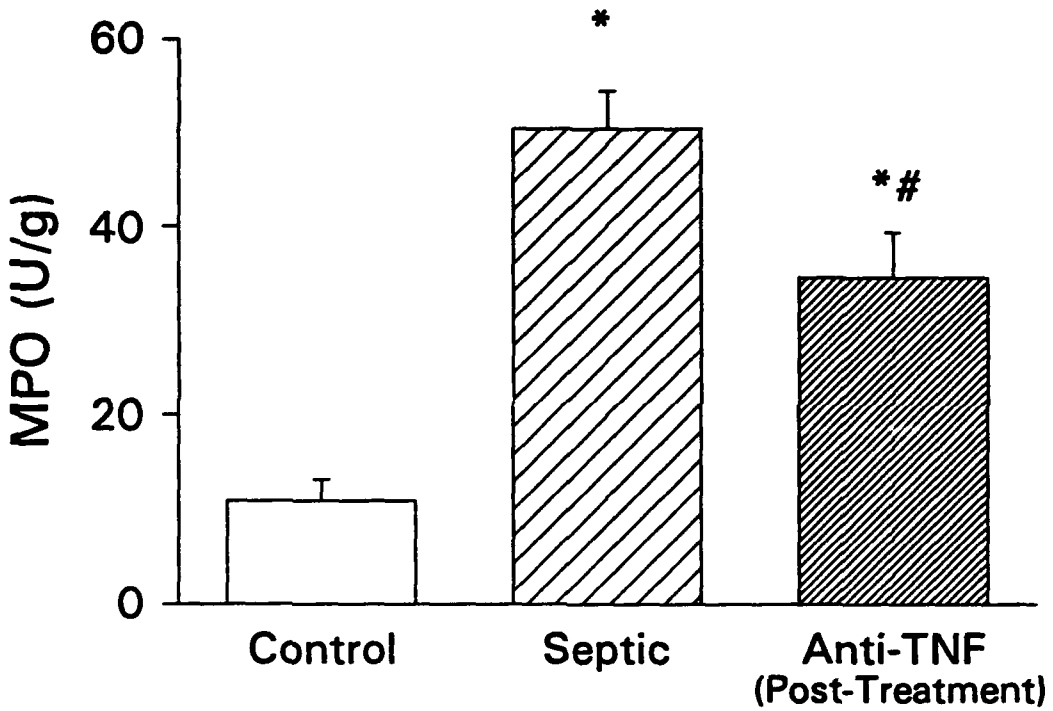
Effects Of Delayed Anti-TNF Antibody On Blood PMN Counts. Sixty min following onset of sepsis anti-TNF antibody (5 mg/kg) was administered. A severe neutropenia was present at the time of treatment. From the point of administration, however, PMN counts began to rise returning to near control levels.

Figure 43



Effects of Delayed Anti-TNF-Alpha Administration On Neutrophil CD11/CD18 expression. When administered to septic animals at 60 min anti-TNF antibody produced an attenuated burst of adhesion receptor expression over the course of 5 hours of sepsis.

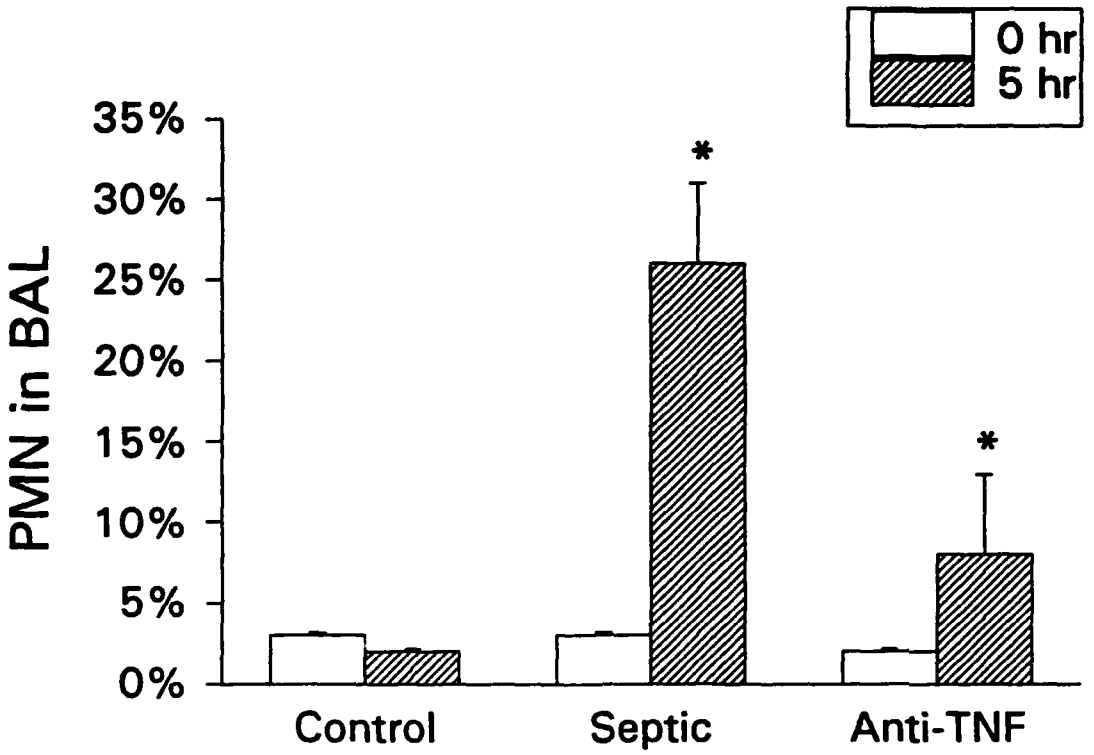
Figure 44



* $p<0.05$ vs Control # $p<0.05$ vs Septic

Myeloperoxidase Analysis of Whole Lung Homogenates. Anti-TNF antibody when administered at 60 minutes following onset of sepsis resulted in increased concentrations of myeloperoxidase in lung homogenates although this value was less when compared to septic untreated animals.

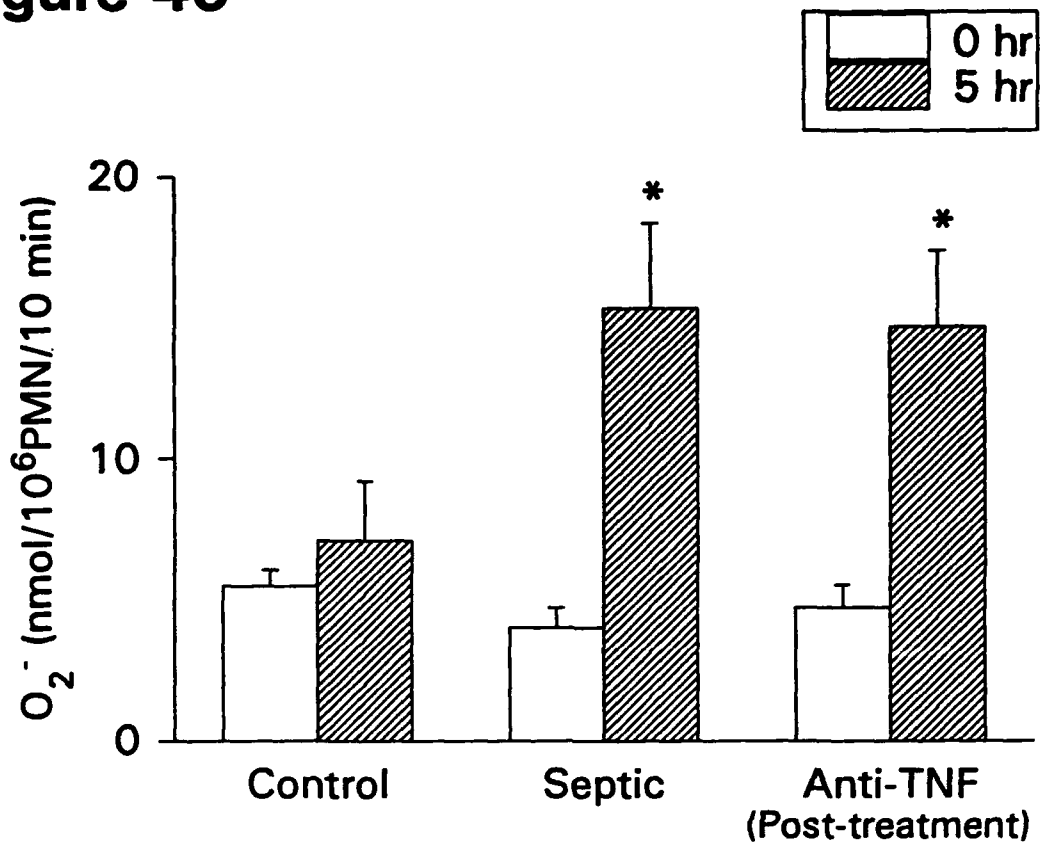
Figure 45



*p<0.05 vs 0 hr

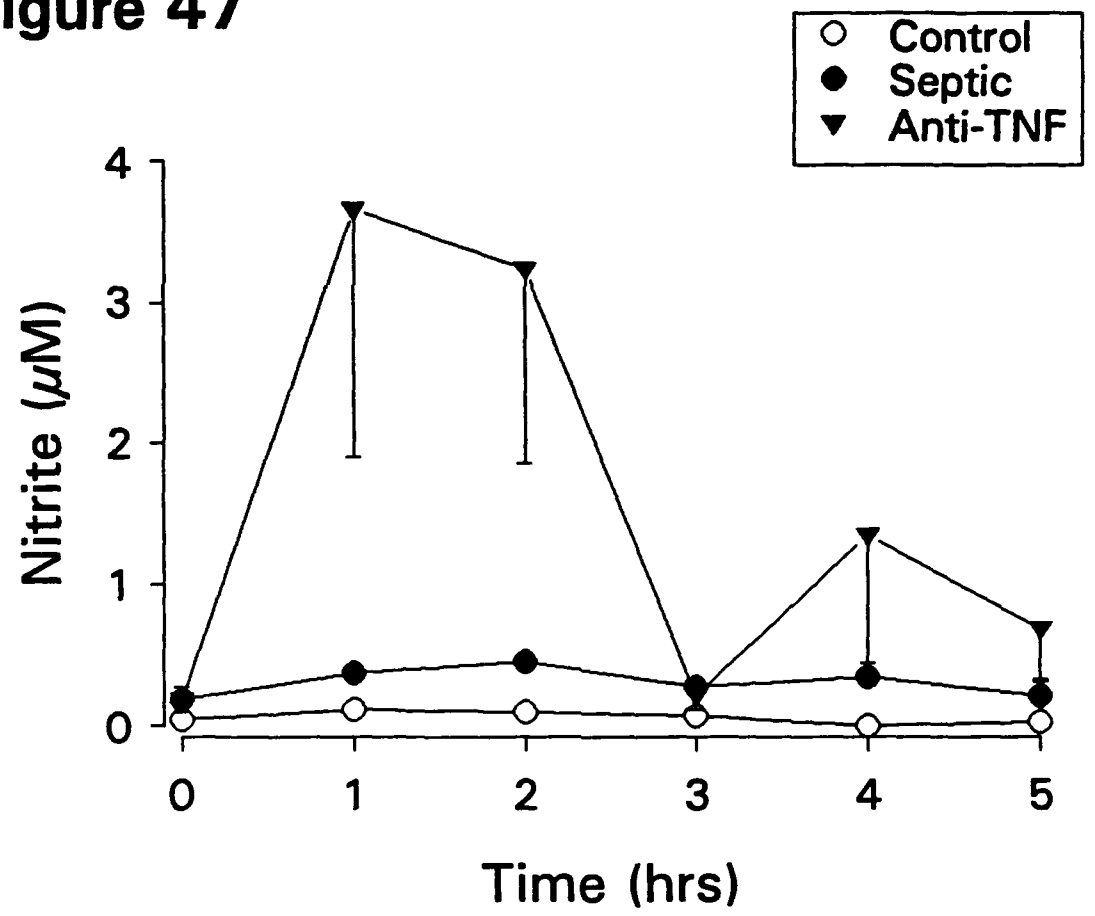
Bronchoalveolar Lavage PMN, Effects of Delayed Anti-TNF Antibody Administration. When administered at 60 minutes, anti-TNF antibody significantly reduced neutrophil migration into the airspaces of the lung.

Figure 46



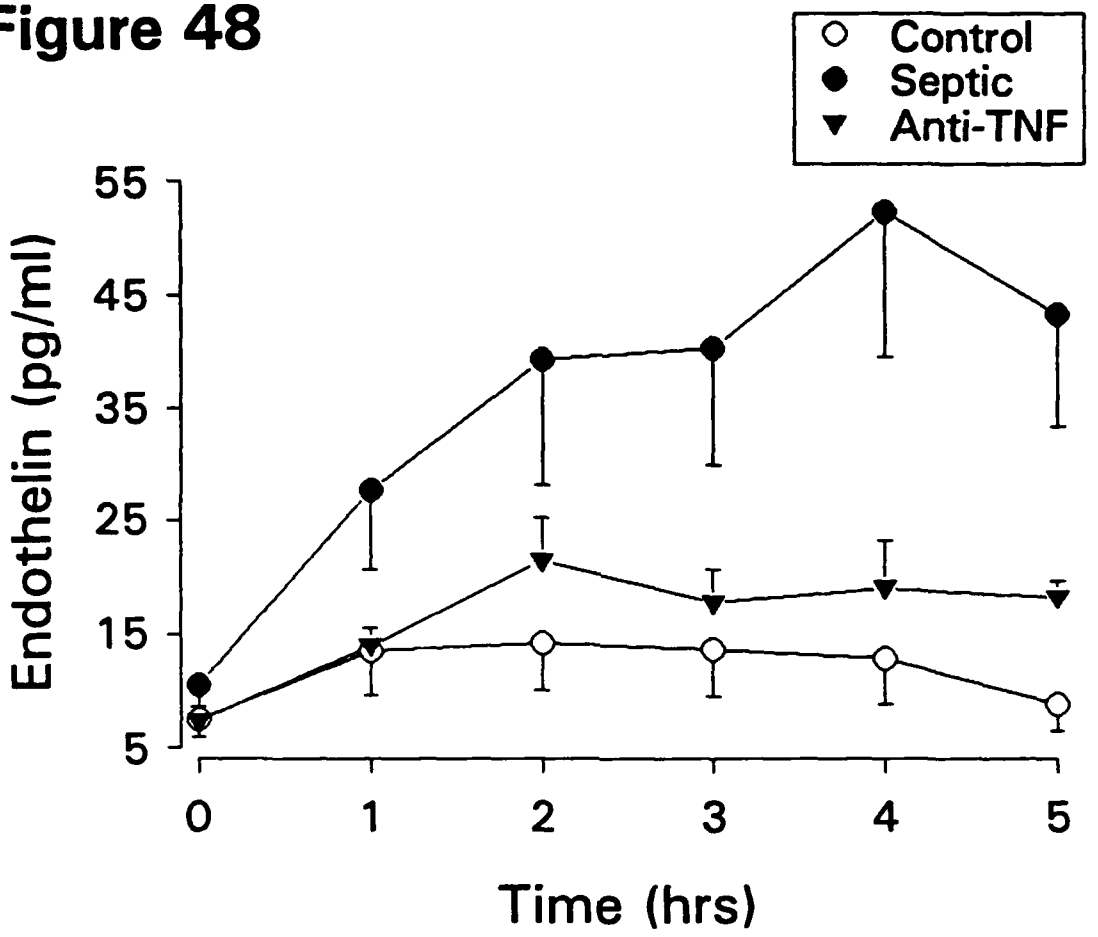
Neutrophil Superoxide Anion Production. When administered at 60 minutes, anti-TNF antibody failed to suppress neutrophil short lived oxidant burst or priming.

Figure 47



Circulating Nitrite Levels. Blood nitrite levels in septic animals pretreated with anti-TNF antibody before sepsis exhibited dramatic increases in the of first 120 min of sepsis. When compared to sepsis Is alone the levels were dramatically different.

Figure 48



Blood Endothelin-1 Levels. Following onset of sepsis levels of endothelin-1 rose dramatically in septic animals. Animals treated with anti-TNF antibody. exhibited significantly attenuated levels.

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36 Windsor ACJ, Mullen PG, Walsh CJ, Fisher BJ, Blocher CR, Fowler AA, Sugerman HJ. Delayed TNFα blockade attenuates pulmonary dysfunction and metabolic acidosis associated with experimental gram negative sepsis. - American College of Surgeons, Committee on Trauma, Virginia State Competition. Washington DC, USA. November 1992. **(Awarded First Place).**

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38 Drenning D, Han J, Mullen PG, Windsor ACJ, Bechard DE, Fowler AA, Vetrovic GW. The role of TNF in granulocyte activation during percutaneous transluminal coronary angioplasty. - Virginia Chapter, American College of Physicians, Richmond, USA. March 1993 .

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American College of Surgeons, Committee on Trauma Competition, National Finals, San Diego California USA. March 1993 - **(Awarded Second Place)**.

40 Leeper-Woodford SK, Fisher BJ, Sugerman HJ, Fowler AA. Diphenhydramine increases TNF activity of pulmonary macrophages. FASEB New Orleans, Louisiana, USA, March 1993

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43 Windsor ACJ, Mullen PG, Walsh CJ, Fisher BJ, Blocher CR, Fowler AA, Sugerman HJ. TNF monoclonal antibody and gram negative sepsis. - American College of Surgeons, Virginia Chapter, Williamsburgh, Virginia, USA. April 1993

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50 Sessler CN, Windsor ACJ, Schwartz M, Watson L, Fowler AA. Soluble ICAM levels are raised in human septic shock. - American College of Chest Physicians, Orlando, Florida, USA (Accepted)